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Jed FAHEY et al.

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Group Art Unit: 1302-1761

For: METHOD OF PREPARING A FOOD PRODUCT FROM  
CRUCIFEROUS SEEDS

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Serial No. 08/528,858

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Respectfully submitted,

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Date

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Sheet 1 of 1		FORM PTO		U. S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTY DOCKET NO. 046585/0102		SERIAL NO. 08/528,858		5,725,895			
LIST OF REFERENCES CITED BY APPLICANT(S) (Use several sheets if necessary)						APPLICANT FAHEY et al.			FILING DATE September 15, 1995		GROUP 1302		
U.S. PATENT DOCUMENTS													
*EXAMINER INITIAL		DOCUMENT NUMBER		DATE		NAME		CLASS		SUBCLASS		FILING DATE IF APPROPRIATE	
FOREIGN PATENT DOCUMENTS													
		DOCUMENT NUMBER		DATE		COUNTRY		CLASS		SUBCLASS		TRANSLATION YES NO	
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		A1		E. Munroe, Sprouts To Grow and Eat, The Stephen Greene Press, Brattleboro, Vermont, pp. 2-9, 14, and 15, Second printing December 1977.									
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		A3		K. Whyte, The Complete Sprouting Cookbook, Troubador Press, San Francisco, 1973.									
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## Design and Synthesis of Bifunctional Isothiocyanate Analogs of Sulforaphane:<sup>†</sup> Correlation between Structure and Potency as Inducers of Anticarcinogenic Detoxication Enzymes<sup>‡</sup>

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Thirty-five bifunctional isothiocyanates were synthesized as structural analogs of sulforaphane [(−)-1-isothiocyanato-4(*R*)-(methylsulfinyl)butane] that was recently isolated from broccoli as the principal and very potent inducer of detoxication (phase 2) enzymes in mouse tissues and murine hepatoma cells (Hepa 1c1c7) in culture (Zhang, Y.; Talalay, P.; Cho, C.-G.; Posner, G. H. *Proc. Natl. Acad. Sci. U.S.A.* 1992, 89, 2399–2403). Determination of the potency of each analog in inducing NAD(P)H:quinone reductase, a phase 2 detoxication enzyme, has allowed generalizations concerning the relation of structure and activity. The most potent analogs were bifunctional derivatives in which the isothiocyanate group was separated from a methylsulfonyl or an acetyl group by three or four carbon atoms, and in some of which these groups were conformationally restricted. Among these analogs, the bicyclic ketoisothiocyanate (±)-*exo*-2-acetyl-6-isothiocyanatonorbornane (30) was a very potent inducer (comparable to sulforaphane) of quinone reductase in hepatoma cells, and it also induced both quinone reductase and glutathione transferases in several mouse organs *in vivo*. This and related bicyclic ketoisothiocyanates represent potent phase 2 enzyme inducers that are relatively easily synthesized and that may be more stable metabolically than the natural sulfoxide sulforaphane.

The fate of chemical carcinogens *in vivo* is determined at least in part by the balance between phase 1 enzymes (cytochromes P-450) that activate many carcinogens to highly reactive electrophilic metabolites capable of damaging DNA and phase 2 enzymes (e.g. glutathione transferases, NAD(P)H:quinone oxidoreductase [QR], UDP-glucuronosyltransferases) that convert these reactive electrophiles to less toxic and more easily excretable products.<sup>1–3</sup> A wide variety of protectors against chemical carcinogenesis are also inducers of phase 2 enzymes in many animal cells and tissues, and there is convincing evidence that monofunctional induction of phase 2 enzymes is a major mechanism responsible for such protection. It is therefore of interest that vegetables, and especially crucifers, are rich in inducer activity and contain a variety of inducer molecules. By the use of a simple screening procedure involving measurement of quinone reductase activities of murine hepatoma cells grown in microtiter plates,<sup>4,5</sup> we have recently isolated and identified sulforaphane [(−)-1-isothiocyanato-4(*R*)-(methylsulfinyl)-butane] as the principal and very potent phase 2 enzyme inducer from SAGA broccoli.<sup>6</sup> A number of natural and synthetic isothiocyanates have been shown to block the neoplastic activity of a variety of carcinogens in rodents, to induce phase 2 enzymes *in vivo* and in cells in culture, and to inhibit metabolic activation of certain carcinogens.<sup>7</sup> In an effort to understand the unusually high potency of

sulforaphane as an enzyme inducer, we designed, synthesized, and evaluated the activities *in vitro* of a number of isothiocyanates, each carrying one additional polar group. We also determined the inducer potency in mouse tissues of (±)-*exo*-2-acetyl-6-isothiocyanatonorbornane (30), one of the most potent synthetic analogs of sulforaphane developed in this study.

### Results

**Acyclic Analogs of Sulforaphane in Which the Methylsulfinyl Group Has Been Replaced by Other Polar Groups.** We have recently shown that sulforaphane, isolated from broccoli, is an extremely potent inducer of QR, equivalent in potency to synthetic racemic sulforaphane.<sup>6</sup> The concentration of sulforaphane required to double the QR activity (CD value) was 0.2 μM. A limited structure-activity study of the analogs CH<sub>3</sub>S-(O)<sub>*m*</sub>(CH)<sub>*n*</sub>N=C=S, where *m* = 0, 1, or 2 and *n* = 3, 4, or 5, led to the following conclusions: (a) sulforaphane is the most potent inducer; (b) the sulfoxides and sulfones do not differ much in potency, but they are more potent than the sulfides; and (c) compounds with four or five methylene groups bridging the methylsulfur and isothiocyanate functions are more potent than those containing only three methylene groups.<sup>6</sup>

The polar sulfoxide group of sulforaphane (CD = 0.2 μM) is clearly very important for inducer activity, since *n*-hexyl isothiocyanate in which the sulfoxide functionality is replaced by a methylene group is much less potent (CD = 15 μM; Table 1). Measurements of the inducer potencies (Table 1) of analogs of the type Z(CH<sub>2</sub>)<sub>*n*</sub>N=C=S, synthesized for these studies, support the following conclusions: (a) of the eight synthetic analogs, only two are very potent; (b) of these two, the methyl ketone 4 and the dimethylphosphine oxide 8 are almost equal in potency to sulforaphane; and (c) whereas the methyl ketone analog

<sup>†</sup> These bifunctional isothiocyanates are the subject of a pending U.S. patent application.

<sup>‡</sup> Abbreviations and trivial names: QR, quinone reductase [NAD(P)H: (quinone-acceptor) oxidoreductase, EC 1.6.99.2]; GST, glutathione *S*-transferase, EC 2.5.1.18; CD value, the concentration of an inducer required to double the specific activity of quinone reductase in Hepa 1c1c7 murine hepatoma cells; DCNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene.

<sup>\*</sup> Department of Chemistry.

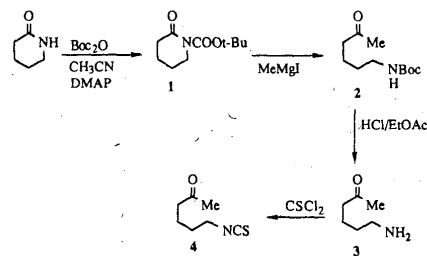
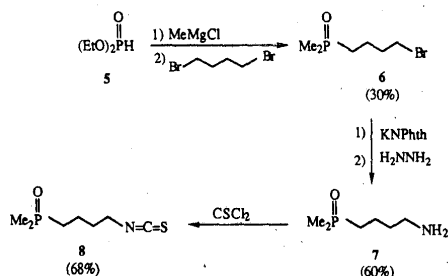
<sup>§</sup> Department of Pharmacology and Molecular Sciences.

<sup>||</sup> Abstract published in *Advance ACS Abstracts*, December 1, 1993.

## Isothiocyanate Analogs of Sulforaphane

**Table 1.** Effect of Replacing the Methylsulfinyl Group of Sulforaphane on Inducer Potency for QR in Murine Hepatoma Cells

Z-CH <sub>2</sub> -CH <sub>2</sub> -CH <sub>2</sub> -N=C=S			
Z	CD	Z	CD
Et	15.0	MeOOC	2.8
CH <sub>3</sub> S(O) (sulforaphane)	0.2	MeSCO	2.8
N=C	2.0	MeCO	0.2
HOOC	2.2	n-BuCO	2.0
CH <sub>3</sub> S(O)CH=CH(CH <sub>2</sub> ) <sub>2</sub> NCS (sulforaphene)	0.4	Me <sub>2</sub> P(=O)	0.4

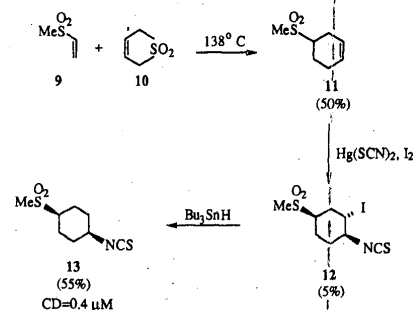
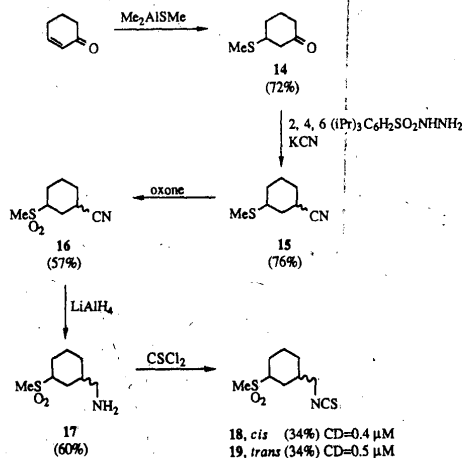
**Scheme 1****Scheme 2**

4 is a potent inducer, the corresponding *n*-butyl ketone is not a good inducer. At present, we do not understand the reason(s) for the differences in potencies among these analogs. The methods of preparation of the most potent analogs are outlined in Schemes 1 and 2.

**Nonaromatic Cyclic Analogs with Restricted Conformations. A. Monocyclic Analogs.** Cyclohexyl isothiocyanate is a relatively weak inducer (CD = 56  $\mu$ M). Using general literature procedures, we prepared three methylsulfonyl isothiocyanates (13, 18, 19) in which the two polar functionalities are separated by four carbon atoms and in which a cyclohexane ring restricts the conformational mobility of the two functional groups (Schemes 3 and 4). Sulfones were targeted because they are more stable than sulfoxides toward several of the reaction conditions used in Schemes 3 and 4 and also because the sulfone erysolin, CH<sub>3</sub>SO<sub>2</sub>(CH<sub>2</sub>)<sub>4</sub>NCS, is only about 2-fold less potent than the sulfoxide sulforaphane.<sup>8</sup> The CD values of these conformationally restricted synthetic sulfonyl isothiocyanates 13, 18, and 19 ranged from 0.4 to 0.5  $\mu$ M, indicating considerable potency and emphasizing the importance of the presence of the polar sulfonyl group, although not its spatial relation to the isothiocyanate function.

**B. Bicyclic Analogs.** Commercial *exo*-norbornyl isothiocyanate (CD = 32  $\mu$ M) is a somewhat more potent

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**Scheme 3****Scheme 4**

inducer than cyclohexyl isothiocyanate (CD = 56  $\mu$ M). The dramatic increase in inducer potency observed by the addition of a methylsulfonyl group to the six-membered monocyclic system was also observed in the bicyclic norbornane analogs. Starting with commercially available 5-acetyl-2-norbornene or easily made 5-(methylsulfonyl)-2-norbornene, we added the elements of H-SCN across the strained carbon-carbon double bond of these norbornenes<sup>8</sup> to produce directly as the major products the mixture of positional and orientational isomers shown in Scheme 5. Chromatographic separations provided the pure bifunctional products listed in Table 2. Assignment of position and orientation of the two functional groups in each product was based on <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy (see the Experimental Section); single-crystal X-ray crystallography confirmed the structure of bifunctional norbornane 23.

Several aspects of the data in Table 2 are noteworthy: (1) 9 of the 12 bifunctional compounds in this table have CD values of less than 1.0, indicative of high enzyme induction potency; (2) where direct comparison is possible, the compounds with the *exo*-oriented Z-substituents are more potent than those with *endo*-oriented Z-substituents (i.e. 29 > 20, 30 > 22); and (3) as was found in the acyclic series (cf. methyl ketone 4), the methyl ketone (i.e. acetyl) functionality in bicyclic analogs 22, 23, and 30 contributes significantly to making these bifunctional compounds very potent inducers.

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Scheme 5

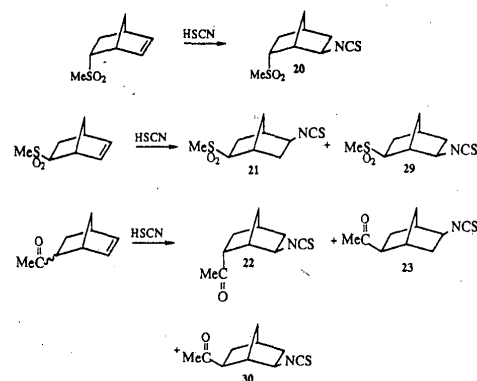
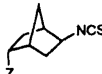
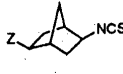
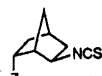
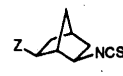


Table 2. Potency of Norbornyl Isothiocyanates in Inducing QR in Murine Hepatoma Cells

Four-Carbon Link between Functionalities					
					
compd	endo Z	CD (μM)	compd	exo Z	CD (μM)
21	MeSO <sub>2</sub>	0.7	23	MeCO	0.4
24	N≡C	0.6	25	O <sub>2</sub> N	1.1
26	MeOOC	0.7	27	MeCH(OH)	0.5
28	OH	19			
Three-Carbon Link between Functionalities					
					
compd	endo Z	CD (μM)	compd	exo Z	CD (μM)
20	MeSO <sub>2</sub>	1.0	29	MeSO <sub>2</sub>	0.2
22	MeCO	0.8	30	MeCO	0.3
			31	MeOOC	1.6

#### Induction of Quinone Reductase and Glutathione Transferase in Mouse Tissues by Keto Isothiocyanate 30.

When synthetic (±)-exo-2-acetyl-6-isothiocyanatonorbornane (30) was administered to female CD-1 mice by gavage in doses of 7.5, 15, or 30 μmol daily for 5 days, the QR and GST (measured with both 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene), specific activities of the cytosols of liver, forestomach, glandular stomach, and proximal small intestine were increased in a dose-dependent manner (Figure 1). The increases in specific activities at the highest doses were generally about 2–4-fold, except for the inductions of GST in the small intestine (measured with DCNB) which were considerably higher (14–16-fold). We conclude that the keto isothiocyanate 30 induces QR not only in murine hepatoma cells, but also, like sulforaphane,<sup>6</sup> induces QR and GST activities in a number of murine organs. Insofar as quantitative comparisons can be made, keto isothiocyanate 30 and sulforaphane do not differ much in inducer potencies or organ-response patterns.

**Aromatic Analogs. A. Aryl Isothiocyanates.** As expected based on previous negative enzyme induction

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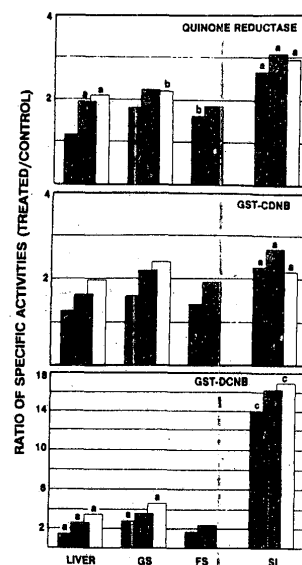


Figure 1. Effect of oral administration of exo-2-acetyl-6-isothiocyanatonorbornane (30) on the specific activities of cytosolic glutathione transferases measured with CDNB (GST-CDNB) and DCNB (GST-DCNB) and quinone reductase of liver, glandular stomach (GS), forestomach (FS), and proximal small intestine (SI) of mice. The compound was administered to 6-week-old female CD-1 mice in 0.1 mL of Emulphor EL620P (GAF, Linden, NJ) daily for 5 days in the following quantities: 7.5 μmol (solid bar), 15 μmol (hashed bar), or 30 μmol (open bar). Four or five animals were studied in each of the treated groups and 10 in the control group. The results are expressed as the ratios (± SEM) of the specific activities of organ cytosols from treated to controls receiving the vehicle only. Cytosols were prepared from the tissues 24 h after the last treatment and assayed for enzyme activities. The enzyme specific activities (nmol·min<sup>-1</sup>·mg<sup>-1</sup> ± SEM) of the cytosols of control mice were as follows. Liver: GST-CDNB, 1080 ± 51.9; GST-DCNB, 10.9 ± 0.78; QR, 57.3 ± 4.1. Glandular stomach: GST-CDNB, 908 ± 27.5; GST-DCNB, 4.5 ± 0.18; QR, 2630 ± 190. Forestomach: GST-CDNB, 1780 ± 57.2; GST-DCNB, 7.6 ± 0.60; QR, 1040 ± 62.9. Small intestine: GST-CDNB, 685 ± 57.9; GST-DCNB, 0.89 ± 0.23; QR, 488 ± 45.9. The relative standard errors of the induction ratios [(SEM/mean) × 100] are as follows: no designation, ± 0–10%; a, ± 10–20%; b, ± 20–30%. The unknown reasons, the forestomachs of mice receiving 30 μmol of 30 were significantly heavier (89.4 ± 3.9 mg, wet weight) than the controls (42.4 ± 2.5 mg) or the forestomachs of mice receiving either 7.5 or 15 μmol of 30 (mean of 46.9 mg). For cytosols of the forestomach homogenates of animals receiving 30 μmol of 30 also contained more protein than the other groups. The specific activities of QR and GST in the cytosols of forestomachs of mice receiving the 30-μmol dose were considerably lower than those treated with the lower doses of 30 and approached control values. The results obtained with the 30-μmol dose are omitted from the figure.

results with phenyl isothiocyanate,<sup>3</sup> none of the *ortho*-substituted phenyl isothiocyanates we have prepared (including *o*-OMe, -Cl, -CH<sub>2</sub>CH<sub>2</sub>SMe) has any significant inducer potency under standard assay conditions.

**B. Benzylic Isothiocyanates.** Prompted by a report by Kjaer on *o*-methoxybenzyl isothiocyanate,<sup>9</sup> we prepared a series of benzylic isothiocyanates substituted in the *ortho* position by the following substituents (CD values [μM] in parentheses): CH<sub>2</sub>CH<sub>3</sub> (4.3), OMe (2.4), NMe<sub>2</sub> (12.5), SMe (6.8), F (13.1), Cl (14.1), Br (12.5), Me (3.7), Et (12.5),



## Isothiocyanate Analogs of Sulforapha

OEt (2.5). None of these analogs was a particularly active inducer or even better than benzyl isothiocyanate itself ( $CD = 2-3 \mu M$ ).

**Conclusion.** In conclusion, several easily prepared, conformationally restricted, non-sulfoxide, nonaromatic, bifunctional analogs of sulforaphane have been identified and have been shown *in vitro* to possess high phase 2 enzyme inducer potency. One of the most promising lead compounds, bicyclic keto isothiocyanate 30, was shown also *in vivo* to induce QR and GST activities in a number of murine organs. Such keto isothiocyanates, probably more stable toward biological oxidation and reduction than sulfoxide-isothiocyanates like sulforaphane, may be useful for protection against cancer.

## Experimental Section

**General Methods.** Thiophosgene was purchased from Carbolabs Inc. (Bethany, CT), and all other reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) and, unless otherwise specified, were used as received without further purification. Benzyl isothiocyanate, obtained from the Aldrich Chemical Co., was distilled under reduced pressure prior to use. Other isothiocyanates which were not synthesized for this project were obtained from Trans World Chemicals (Rockville, MD).  $^1H$  and  $^{13}C$  NMR spectra were determined on a Bruker AMX 300 MHz or a Varian XL-400 MHz spectrometer. High-resolution mass spectra were obtained at 70 eV on a VG-70S mass spectrometer. FT-IR spectra were recorded on a Perkin-Elmer Model 1600 FT-IR spectrophotometer. Elemental analyses were performed by Atlantic Microlab, Norcross, GA. Medium-pressure column chromatography was performed with silica gel (EM SCIENCE, 230-400 mesh). High-pressure liquid chromatography was performed with RAININ HPXL chromatography using a semi-prep silica column.

**Boc-Protected Lactam 1.** To a flask charged with 0.56 g (5.7 mmol) of  $\delta$ -valerolactam, 60 mg (0.5 mmol) of 4-(dimethylamino)-pyridine (DMAP), and 30 mL of acetonitrile was added 1.22 g (5.7 mol) of di-*tert*-butyl dicarbonate (Boc<sub>2</sub>O) at room temperature (RT). After 4 h at RT, the reaction mixture was concentrated and partitioned between ether and 1 M  $KHSO_4$ . The separated organic layer was washed with saturated  $NaHCO_3$  and brine, dried over  $MgSO_4$ , and concentrated to give 0.90 g of product as a bright yellowish green liquid (used for the next reaction without purification).

**4-Boc-aminobutyl Methyl Ketone (2).** To protected lactam 1 in 10 mL of THF was added 2 mL of  $MeMgI$  (3 M in ether, 6 mmol) slowly at  $-78^\circ C$ . After 3 h at  $-78^\circ C$ , the reaction mixture was quenched with aqueous  $NH_4Cl$ , and the resulting solution was extracted with ether (2  $\times$  20 mL). The combined ether solution was washed with brine, dried over  $MgSO_4$ , and concentrated in vacuo to give crude methyl ketone 2 as a brown oil (used for the next reaction without further purification).

**4-Aminobutyl Methyl Ketone (3).** To methyl ketone 2 dissolved in 3 mL of EtOAc was added 1 mL of 37% of HCl at RT. After 30 min, the reaction mixture was diluted with 5 mL of  $H_2O$  and washed with ether. The aqueous solution was then strongly basified with solid NaOH and extracted with  $CHCl_3$  (2  $\times$  20 mL). The organic solution was dried over  $K_2CO_3$  and concentrated to give a smelly light brown oil (used for the next reaction without purification).

**2-Oxohexyl Isothiocyanate (4).** To amino methyl ketone 3 in 2 mL of  $H_2O$  and 2 mL of  $CHCl_3$  were added 0.16 mL of  $CSCl_2$  (1.58 mmol) and 2 mL of 5% NaOH at RT. After 30 min the reaction mixture was diluted with 10 mL of  $CHCl_3$ , and the decanted organic layer was dried over  $MgSO_4$ , concentrated, and chromatographed (8/2 Hex/EtOAc) to give 42 mg of isothiocyanate 4 as a brown oil in an overall yield of 6% from lactam 1:  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  3.54–3.49 (m, 2H), 2.53–2.46 (m, 2H), 2.16 (s, 3H), 1.72–1.68 (m, 4H); FT-IR ( $CHCl_3$ ) 3019, 2191, 2112, 1715, 1224  $cm^{-1}$ ;  $^{13}C$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  207.8, 44.8, 42.4, 29.9, 29.3, 20.6; HRMS calcd for  $C_7H_{11}NOS$  157.0561, found 157.0565.

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**(4-Bromobutyl)dimethylphosphine Oxide (6).** To a 25-mL flame-dried round-bottomed flask charged with 15.2 mL (45.6 mmol) of  $MeMgCl$  (3.0 M in THF) was added 1.5 mL (11.41 mmol) of diethyl phosphite (5) while the internal temperature was maintained around  $25^\circ C$  with occasional cooling with an ice-water bath. After 1 h, the mixture was cannulated into a separate flask charged with 2.55 mL (22.82 mmol) of 1,4-dibromobutane and 15 mL of THF at  $0^\circ C$  under an Ar atmosphere. Upon addition, the reaction mixture was heated under reflux for 5 h, cooled, and dumped into 30 mL of cold dilute HCl. The resulting aqueous solution was extracted with  $CHCl_3$  (3  $\times$  50 mL), and the combined organic solution was washed with saturated  $K_2CO_3$ , dried over  $K_2CO_3$ , and concentrated in vacuo to give 2.48 g of crude product as a tan oil. Purification by flash column chromatography (silica gel, 8/2 EtOAc/MeOH  $\rightarrow$  6/4 EtOAc/hexane) afforded 0.72 g of phosphine oxide 6 as a colorless oil (used for the next reaction without purification).

**(4-Aminobutyl)dimethylphosphine Oxide (7).** In a 100-mL round-bottomed flask were placed 0.733 g (3.44 mmol) of phosphine oxide 6, 0.766 g of potassium phthalimide, and 20 mL of DMF. The mixture was heated under reflux for 4 h, cooled, and dumped into 60 mL of  $CHCl_3$ . The organic solution was washed with  $H_2O$ , dried over  $NaHCO_3$ , and concentrated in vacuo to afford 0.92 g of product phthalimide as a white solid. To a separate flask charged with 0.10 g of product phthalimide was added 4 mL of methanolic hydrazine (0.2 M in MeOH) at RT. After 14 h at RT, the reaction mixture was concentrated, and the residue was treated with 5 mL of 1 N HCl, washed with  $CHCl_3$ , and strongly basified with solid NaOH. The basified solution was then extracted with  $CHCl_3$  (2  $\times$  20 mL), and the combined organic solution was dried over  $K_2CO_3$  and concentrated in vacuo to give 33 mg of amine phosphine oxide 7 as a white solid (used for the next reaction without further purification).

**(4-Isothiocyanatobutyl)dimethylphosphine Oxide (8).** To a flask charged with 83 mg (0.22 mmol) of amine 7 and 1 mL of  $CHCl_3$  were added at RT 0.02 mL (0.27 mmol) of  $CSCl_2$  and 0.3 mL of 1 N NaOH. After 35 min at RT, the reaction mixture was partitioned between 10 mL of  $CHCl_3$  and 10 mL of  $H_2O$ . The separated organic layer was dried over  $MgSO_4$ , concentrated in vacuo, and chromatographed (8/2 EtOAc/MeOH) to afford 29 mg of isothiocyanate 8 as a reddish yellow oil in 68% yield:  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  3.54 (t,  $J = 6.0$  Hz, 2H), 1.82–1.70 (m, 6H), 1.48 (s, 3H), 1.44 (s, 3H); FT-IR ( $CHCl_3$ ) 2941, 2191, 2097, 1302, 1173  $cm^{-1}$ ;  $^{13}C$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  44.5, 30.6 (d,  $J = 20.2$  Hz, 1C), 30.7 (d,  $J = 34.7$  Hz, 1C), 19.3, 16.2 (d,  $J = 69$  Hz, 2C);  $^{31}P$  NMR ( $CDCl_3$ )  $\delta$  46.1; HRMS calcd for  $C_7H_{14}NOPS$  191.0534, found 191.0536.

**4-(Methylsulfonyl)cyclohexene (11).** In a sealed tube were placed 0.50 g (5.5 mmol) of methyl vinyl sulfone (9), 0.67 g (5.5 mmol) of 1,4-but-2-enediyl sulfone (10) and 2 mL of absolute EtOH. After 2 days at  $138^\circ C$ , the reaction mixture was cooled and poured into aqueous  $Na_2CO_3$ . After 10 min with vigorous stirring, the aqueous solution was extracted with ether (2  $\times$  10 mL), dried over  $MgSO_4$ , concentrated in vacuo, and chromatographed (1/1 hexane/ether) to give 0.20 g (2.2 mmol, 40% recovery) of methyl vinyl sulfone (9) and 0.40 g of sulfone 11 as a brown oil in 50% yield.

**Iodo Isothiocyanate 12.** To a flask charged with 342 mg (0.63 mmol) of  $Hg(SCN)_2$  was added a premixed solution of  $I_2$  in 8 mL of benzene. After 30 min at RT, to this mixture was added 202 mg (1.26 mmol) of cyclohexene 11 dissolved in 1 mL of benzene, and the flask containing the reaction mixture was wrapped with aluminum foil and stirred for 7.5 days at RT under an argon atmosphere. The reaction mixture was then filtered off, and the solid material was washed with ether. The ether solution was washed with aqueous KI, aqueous  $Na_2S_2O_3$ , and brine successively, dried over  $MgSO_4$ , and concentrated in vacuo. Flash column chromatography (1/1 ether/hexane) afforded 20 mg of iodide 12 (5% yield) as a brown oil along with three other isomers (11% yield).

**Sulfonyl Isothiocyanate 13.** To a flask charged with 21 mg (0.07 mmol) of iodide 12 and 1 mL of benzene was added 0.05 mL (0.2 mmol, 3 equiv) of  $Bu_3SnH$  at RT. After 10 h, the reaction mixture was treated with 2 mL of wet ether and 35 mg (0.2 mmol) of DBU (1,8-diazabicyclo[5.4.0]undec-7-ene). The resulting mixture was filtered off, concentrated in vacuo and chromato-

graphed (100% ether  $\rightarrow$  1/1 ether/EtOAc) to afford 7.3 mg of isothiocyanate 13 as white solid (mp 123 °C) in 55% yield:  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  4.11–4.08 (m, 1H), 2.85 (s, 3H), 2.87–2.80 (m, 1H, overlapped), 2.23–2.18 (m, 4H), 1.93 (dd,  $J$  = 14.8, 4.4 Hz, 1H), 1.87 (dd,  $J$  = 13.2, 3.2 Hz, 1H), 1.67 (tt,  $J$  = 13.2, 3.6 Hz, 2H); FT-IR ( $\text{CHCl}_3$ ) 3025, 2943, 2261, 2085, 1302  $\text{cm}^{-1}$ ;  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  133.5, 61.2, 52.3, 36.8, 30.5, 20.6; HRMS calcd for  $\text{C}_9\text{H}_{13}\text{NO}_2\text{S}_2$  219.0388, found 219.0391.

**3-Cyanocyclohexyl Methyl Sulfide (15).** Into a 100-mL round-bottomed flask were placed 0.438 g (3.0 mmol) of 14,<sup>11</sup> 1.418 g (4.8 mmol) of 2,4,6-triisopropylbenzenesulfonyl hydrazide,<sup>10</sup> and 8 mL of MeOH at RT. After 1 h, 0.739 g (11.3 mmol) of KCN was added at RT, and the resulting mixture was heated under gentle reflux for 3 h. The reaction mixture was cooled, diluted with 20 mL of  $\text{H}_2\text{O}$ , and extracted with  $\text{CH}_2\text{Cl}_2$  (2  $\times$  20 mL). The organic solution was washed with aqueous  $\text{NaHCO}_3$ , dried over  $\text{MgSO}_4$ , concentrated in vacuo, and purified by flash column chromatography (8/2 hexane/EtOAc) to afford 0.360 g of sulfide 15 as a yellow oil in 76% yield.

**3-Cyanocyclohexyl Methyl Sulfone (16).** To a flask charged with 0.36 g (2.32 mmol) of sulfide 15 and 10 mL of aqueous MeOH (9/1 v/v MeOH/ $\text{H}_2\text{O}$ ) was added 2.75 g (4.64 mmol) of Oxone (Aldrich,  $2\text{KHSO}_5\cdot\text{KHSO}_4\cdot\text{K}_2\text{SO}_4$ ) at RT. After 24 h, the reaction mixture was filtered through a sintered-glass funnel, and the filtered solid material was washed with 50 mL of  $\text{CHCl}_3$ . The combined organic solution was washed with  $\text{H}_2\text{O}$ , dried over  $\text{MgSO}_4$ , and concentrated in vacuo to afford 0.246 g of sulfone 16 (57% yield) as a colorless oil. This material was used in the next reaction without purification.

**3-(Aminomethyl)cyclohexyl Methyl Sulfone (17).** To a suspension of 0.098 g (2.59 mmol) of  $\text{LiAlH}_4$  in 10 mL of anhydrous ether was cannulated 0.246 g (1.31 mmol) of nitrile 16 dissolved in 3 mL of THF at RT. Upon addition, the reaction mixture was heated under reflux. After 2.5 h, the reaction mixture was cooled, quenched with 0.5 mL of  $\text{H}_2\text{O}$  and 0.5 mL of 5% NaOH, and filtered through a sintered-glass funnel. The solid material filtered was thoroughly washed with ether. The combined organic solution was dried over  $\text{K}_2\text{CO}_3$  and concentrated in vacuo to afford 0.150 g of amine 17 (60% yield) as a colorless oil. This material was used in the next reaction without purification.

**cis-(3-(Methylsulfonyl)cyclohexyl)methyl Isothiocyanate 18 and trans-(3-(Methylsulfonyl)cyclohexyl)methyl Isothiocyanate 19.** To a flask charged with 0.15 g (0.78 mmol) of amine 17 and 3 mL of  $\text{CHCl}_3$  were added 0.07 mL (0.92 mmol) of  $\text{CSCl}_2$  and 1.5 mL of 5% NaOH at RT. After 1 h, the reaction mixture was diluted with 10 mL of  $\text{CH}_2\text{Cl}_2$ , washed with  $\text{H}_2\text{O}$  and brine, dried over  $\text{MgSO}_4$ , concentrated in vacuo, and chromatographed (1/1 hexane/EtOAc) to give 0.123 g of products (67% yield) as a mixture of isothiocyanates 18 and 19 (1:1 ratio). HPLC (40/60 EtOAc/hexane) separation afforded analytically pure 18 and 19 (both as a colorless oil). 18:  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.47 (d,  $J$  = 6.0 Hz, 2H), 2.92–2.82 (m, 1H), 2.84 (s, 3H), 2.28–2.20 (m, 2H), 2.04 (tt,  $J$  = 6.8, 3.0 Hz, 1H), 1.87–1.75 (m, 2H), 1.53–1.27 (m, 3H), 1.06 (tq,  $J$  = 12.2, 3.6 Hz, 1H); FT-IR ( $\text{CHCl}_3$ ) 3025, 2931, 2861, 2191, 2097, 1449, 1308  $\text{cm}^{-1}$ ;  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  56.5, 45.5, 32.6, 32.5, 23.9, 23.8, 20.0, 19.1; HRMS calcd for  $\text{C}_9\text{H}_{13}\text{NO}_2\text{S}_2$  233.0544, found 233.0548. 19:  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.50 (d,  $J$  = 6.8 Hz, 2H), 3.09–3.03 (m, 1H), 2.88 (s, 3H), 2.45–2.37 (m, 1H), 2.14–2.07 (m, 1H), 1.98–1.84 (m, 4H), 1.74–1.66 (m, 1H), 1.59–1.41 (m, 2H); FT-IR ( $\text{CHCl}_3$ ) 3013, 2943, 2872, 2191, 2097, 1449, 1308  $\text{cm}^{-1}$ ;  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  52.6, 43.5, 33.6, 28.0, 22.5, 22.1, 19.5, 14.9; HRMS calcd for  $\text{C}_9\text{H}_{13}\text{NO}_2\text{S}_2$  233.0544, found 233.0545.

**2-endo-Methylsulfonyl Isothiocyanate 20.** In a 20-mL hydrolysis tube were placed 349 mg (2.03 mmol) of 5-endo-(methylsulfonyl)-2-norbornene,<sup>12</sup> 394 mg (4.05 mmol) of KSCN, 319 mg (3.25 mmol) of  $\text{H}_2\text{SO}_4$ , and 0.1 mL of  $\text{H}_2\text{O}$  at RT via literature precedent.<sup>13</sup> The tube was sealed and shaken vigorously for 5 min to give a finely divided yellow suspension. After 4 days at 50 °C, the reaction mixture was filtered through a sintered-glass funnel, and the brown solid was washed with ether. The combined organic solution was washed with  $\text{H}_2\text{O}$ , brine, dried over  $\text{MgSO}_4$ , and concentrated to give 270 mg of crude product containing unreacted starting material as the major component. Flash column chromatography (1/1 hexane/EtOAc) afforded 47 mg of isothiocyanate 20 as a white solid. Recrystallization from

a 2:1 mixture of hexane and ether gave 23 mg of isothiocyanate 20 (5% yield) as white needles (mp 110–111 °C). The *endo* orientation of the methylsulfonyl group was assigned based on its  $^1\text{H}$  NMR chemical shift ( $\delta$  2.84) being upfield from that of the corresponding *exo* methylsulfonyl groups of 21 and 28 ( $\delta$  2.86–2.87).<sup>14</sup> The 1,3-relationship of the two substituents was assigned based on the difference in chemical shifts between the bridgehead hydrogens ( $\Delta\delta$  = 0.2) vs that in the 1,4-positional isomer 21 ( $\Delta\delta$  = 0.1).<sup>15</sup>  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.79 (dd,  $J$  = 7.6, 4.4 Hz, 1H), 3.30–3.25 (m, 1H), 2.90–2.84 (m, 1H), 2.84 (s, 3H), 2.68 (d,  $J$  = 4.4 Hz, 1H), 2.02 (ddd,  $J$  = 13.1, 10.1, 4.2 Hz, 1H), 1.90–1.86 (m, 1H), 1.72–1.52 (m, 4H); FT-IR ( $\text{CHCl}_3$ ) 3 025, 2120, 2097, 1320  $\text{cm}^{-1}$ . Anal. ( $\text{C}_9\text{H}_{13}\text{NO}_2\text{S}_2$ ) C, H, N, S.

**2-exo-Methylsulfonyl Isothiocyanate 21 and 2-exo-Methylsulfonyl Isothiocyanate 29.** The same procedure as described for 20 was used except that the reaction mixture was stirred for 6 days at 65 °C. After workup, isothiocyanates 21 (17% yield) and 29 (5% yield) were isolated by flash column chromatography (100% ether  $\rightarrow$  100% EtOAc). Isothiocyanate 21 was recrystallized from  $\text{CH}_2\text{Cl}_2$ /ether/hexane to afford ivy leaf-shaped crystals (mp 142–143 °C) in 12% yield. Isothiocyanate 29 was recrystallized from ether to afford small needles (mp 82–82.5 °C) in 4% yield. The assignment of positional isomers was done as described for isothiocyanate 20. Isothiocyanate 21:  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.66 (t,  $J$  = 6.8 Hz, 1H), 2.90 (bs, 1H), 2.86 (s, 3H), 2.80 (dd,  $J$  = 8.0, 5.2 Hz, 1H), 2.66 (bd,  $J$  = 5.2 Hz, 1H), 2.12 (td,  $J$  = 14.0, 5.2 Hz, 1H), 2.03 (dt,  $J$  = 12.0, 2.2 Hz, 1H), 1.88–1.84 (m, 2H), 1.68–1.60 (m, 2H); FT-IR ( $\text{CHCl}_3$ ) 3025, 2120, 2073, 1320  $\text{cm}^{-1}$ . Anal. ( $\text{C}_9\text{H}_{13}\text{NO}_2\text{S}_2$ ) C, H, N, S. Isothiocyanate 29:  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.65 (dd,  $J$  = 6.8, 2.8 Hz, 1H), 2.98 (bs, 1H), 2.87 (s, 3H), 2.76 (dd,  $J$  = 6.8, 1.2 Hz, 1H), 2.58 (bs, 1H), 2.06–1.61 (m, 6H); FT-IR ( $\text{CHCl}_3$ ) 3025, 2978, 2191, 2120, 2085, 1349, 1308, 1138  $\text{cm}^{-1}$ ;  $^{13}\text{C}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  132.2, 61.5, 58.0, 45.5, 39.6, 39.2, 35.3, 33.7, 31.2; HRMS calcd for  $\text{C}_9\text{H}_{13}\text{NO}_2\text{S}_2$  231.0388, found 231.0390.

**2-endo-Acetyl Isothiocyanate 22, 2-exo-Acetyl Isothiocyanate 23, and 2-exo-Acetyl Isothiocyanate 30.** To a 100-mL three-neck round-bottomed flask equipped with a magnetic stirring bar, dropping funnel, and reflux condenser were placed 2.0 g (14.7 mmol) of 5-acetyl-2-norbornene (mixture of *endo* and *exo*, Aldrich Chemical Co.), 2.86 g (29.4 mmol) of KSCN, and 10 mL of benzene. To this solution was added at RT a mixture of 2.1 g (21.5 mmol) of concentrated sulfuric acid and 1.0 mL of water slowly using a dropping funnel. After 4 days at 50 °C, the reaction mixture was filtered through a sintered-glass funnel. The filtered white solid was washed with 50 mL of ether. The combined organic solution was then washed with water and brine successively, dried over  $\text{MgSO}_4$ , and concentrated in vacuo to afford a tan oil. Subsequent purification via flash column chromatography (2/8 ether/hexane) afforded 1.73 g of product (60% yield, colorless oil) as a mixture of four stereoisomers. Purification by HPLC (97/3 hexane/EtOAc, 10 mL/min) gave isothiocyanates 22 (10% yield), 23 (23% yield), and 30 (22% yield). The assignment of positional and orientational isomers was done as described for isothiocyanates 20, 21, and 29. The structure of isothiocyanate 23 was confirmed by X-ray crystallography. Isothiocyanate 22:  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.51 (dd,  $J$  = 7.6, 2.8 Hz, 1H), 2.85–2.79 (m, 1H), 2.68–2.54 (m, 1H), 2.43 (d,  $J$  = 4.8 Hz, 1H), 2.06 (s, 3 H), 1.74–1.70 (m, 1H), 1.66 (ddd,  $J$  = 13.6, 7.6, 2.4 Hz, 1H), 1.59–1.43 (m, 4H); FT-IR ( $\text{CHCl}_3$ ) 3013, 2955, 2132, 2097, 1702, 1343  $\text{cm}^{-1}$ . Anal. ( $\text{C}_{10}\text{H}_{13}\text{NOS}$ ) C, H, N, S. Isothiocyanate 23:  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.60 (dd,  $J$  = 7.2, 2.8 Hz, 1H), 2.57 (d,  $J$  = 4.4 Hz, 1H), 2.51 (d,  $J$  = 4.8 Hz, 1H), 2.34 (dd,  $J$  = 8.8,  $J$  = 5.2 Hz, 1H), 2.15 (s, 3H), 1.98 (dt,  $J$  = 13.2, 4.8 Hz, 1H), 1.88 (ddd,  $J$  = 13.2, 7.6, 2.4 Hz, 1H), 1.75 (dt,  $J$  = 13.6, 4.4 Hz, 1H), 1.52 (ddt,  $J$  = 10.8, 4.0, 1.6 Hz, 1H), 1.33 (ddt,  $J$  = 10.8, 4.0, 1.6 Hz, 1H), 1.22 (ddd,  $J$  = 13.2, 8.8, 2.0 Hz, 1H); FT-IR ( $\text{CHCl}_3$ ) 2978, 2179, 2146, 2085, 1708, 1449, 1343  $\text{cm}^{-1}$ . Anal. ( $\text{C}_{10}\text{H}_{13}\text{NOS}$ ) C, H, N, S. Isothiocyanate 30:  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.64 (dd,  $J$  = 7.6, 2.8 Hz, 1H), 2.71 (bs, 1H), 2.43 (dd,  $J$  = 4.5, 3.6 Hz, 1H), 2.31 (dd,  $J$  = 8.4, 6.0 Hz, 1H), 2.17 (s, 3H), 1.83–1.67 (m, 2H), 1.58–1.54 (m, 2H), 1.38–1.30 (m, 2H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  207.4, 130.1, 58.3, 50.7, 46.5, 39.9, 35.4, 33.6, 31.5, 28.7; FT-IR ( $\text{CHCl}_3$ ) 2955, 2132, 2085, 1708, 1343  $\text{cm}^{-1}$ ; HRMS calcd for  $\text{C}_{10}\text{H}_{13}\text{NOS}$  195.0719, found 195.0719.



**2-exo-Cyanonorbornyl Isothiocyanate 24.** To a sealed tube charged with 145 mg (1.2 mmol) of 5-exo-cyano-2-norbornene, 238 mg (2.4 mmol) of KSCN, and 3 mL of benzene were added 177 mg (2.4 mmol) of  $H_2SO_4$  and 0.08 mL of  $H_2O$ . After 48 h at 63 °C, the reaction mixture was diluted with ether, filtered, concentrated, and chromatographed (3/7 ether/hexane) to give 77 mg (53%) of starting material and 33 mg of product as a mixture of two isomers. Subsequent HPLC separation (9/1 hexane/EtOAc) afforded 30 mg of isothiocyanate 24 as a white solid (mp 49.5–50.5 °C from hexane) in 14% yield:  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  3.59 (t,  $J$  = 5.6 Hz, 1H), 2.73 (bs, 1H), 2.64 (d,  $J$  = 4.4 Hz, 1H), 2.32 (ddd,  $J$  = 9.2, 4.8, 1.6 Hz, 1H), 1.90 (dt,  $J$  = 13.6 Hz, 4.8, 1H), 1.85–1.73 (m, 5H); FT-IR ( $CHCl_3$ ) 3021, 2979, 2954, 2240, 2201, 2146, 2100, 1452, 1349  $cm^{-1}$ ;  $^{13}C$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  131.0, 122.2, 57.3, 43.4, 40.8, 39.0, 34.4, 31.8, 29.9; HRMS calcd for  $C_6H_{10}N_2S$  178.0563, found 178.0561.

**2-exo-Nitronorbornyl Isothiocyanate 25.** To a flask charged with 183 mg (1.3 mmol) of 5-exo-nitro-2-norbornene,<sup>10</sup> 255 mg (2.6 mmol) of KSCN, and 6 mL of benzene were added 190 mg (2.6 mmol) of  $H_2SO_4$  and 0.1 mL of  $H_2O$ . After 75 h at 40 °C, the reaction mixture was diluted with ether, filtered off, concentrated, and chromatographed (9/1 hexane/ether) to afford 107 mg of starting material (59%) and 36 mg of isothiocyanate 25 as a yellow solid (14%). Subsequent recrystallization from ether/hexane gave 26 mg of isothiocyanate 25 as colorless needles (mp 67–68 °C) in 10% yield:  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  4.32 (q,  $J$  = 8.0 Hz, 1H), 3.62 (t,  $J$  = 5.2 Hz, 1H), 3.00 (bs, 1H), 2.67 (d,  $J$  = 4.8 Hz, 1H), 2.39 (dt,  $J$  = 14.4, 4.0 Hz, 1H), 1.88–1.71 (m, 5H); FT-IR ( $CHCl_3$ ) 3013, 2132, 2085, 1549, 1367, 1220  $cm^{-1}$ . Anal. ( $C_6H_{10}N_2O_2S$ ), C, H, N, S.

**2-exo-(Methoxycarbonyl)norbornyl Isothiocyanate 26 and 2-exo-(Methoxycarbonyl)norbornyl Isothiocyanate 31.** To a flask charged with 40 mg (0.26 mmol) of 5-exo-(methoxycarbonyl)-2-norbornene,<sup>17</sup> 51 mg (0.53 mmol) of KSCN, and 0.5 mL of benzene were added 38 mg (0.53 mmol) of  $H_2SO_4$  and 0.02 mL of  $H_2O$ . After 46 h at 63 °C, the reaction mixture was directly chromatographed (7/3 hexane/ether) to give 20 mg (0.09 mmol) of product as a mixture of two isomers based on  $^1H$  NMR analysis (26:31 85:15). Purification by HPLC (95/5 hexane/EtOAc) afforded pure isothiocyanates 26 as a white solid (ca. 31% yield, mp 39.5–40.5 °C) and 31 as a liquid (ca. 6% yield). Isothiocyanate 26:  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  3.67 (s, 3H), 3.59 (dd,  $J$  = 7.6, 3.2 Hz, 1H), 2.63 (d,  $J$  = 3.5 Hz, 1H), 2.55 (d,  $J$  = 4.3 Hz, 1H), 2.25 (dd,  $J$  = 8.5, 4.3 Hz, 1H), 1.97 (dt,  $J$  = 13.7, 4.7 Hz, 1H), 1.85 (ddd,  $J$  = 13.4, 7.8, 1.9 Hz, 1H), 1.74 (dt,  $J$  = 13.5, 3.8 Hz, 1H), 1.62–1.53 (m, 2H), 1.42–1.36 (m, 1H); FT-IR ( $CHCl_3$ ) 2976, 2953, 2140, 2088, 1729, 1437, 1346  $cm^{-1}$ ;  $^{13}C$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  175.2, 58.0, 51.9, 45.0, 43.8, 40.2, 40.1, 33.6, 29.8; HRMS calcd for  $C_{10}H_{14}NO_2S$  211.0667, found 211.0669. 31:  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  3.68 (s, 3H), 3.62 (dd,  $J$  = 7.6, 3.6 Hz, 1H), 2.76 (bs, 1H), 2.44 (bs, 1H), 2.22 (dd,  $J$  = 8.4, 5.6 Hz, 1H), 1.88–1.40 (m, 6H); FT-IR ( $CHCl_3$ ) 3027, 3009, 2974, 2954, 2197, 2132, 1732, 1437, 1346  $cm^{-1}$ ;  $^{13}C$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  174.7, 58.2, 52.1, 47.8, 42.8, 39.8, 35.4, 34.0, 33.0; HRMS calcd for  $C_{10}H_{14}NO_2S$  211.0667, found 211.0667.

**exo-2-(1'-Hydroxyethyl)norbornyl Isothiocyanate 27.** To 37.3 mg (0.2 mmol) of acetyl isothiocyanate 23 in 1.5 mL of MeOH was added 8.7 mg (0.2 mmol) of  $NaBH_4$  slowly at 0 °C. After 15 min at 0 °C, the reaction mixture was treated with a few drops of water, diluted with ether, dried over  $MgSO_4$ , concentrated in vacuo, and purified by prep TLC (8/2 ether/hexane) to give 21.0 mg of hydroxy isothiocyanate 27 as a mixture of diastereomers (white solid; mp 64–69 °C recrystallized from hexane) in 56% yield:  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  1.19 (d,  $J$  = 6.0 Hz,  $CH_3$ ), 1.10 (d,  $J$  = 6.4 Hz,  $CH_3$ ); FT-IR ( $CHCl_3$ ) 3623, 3460, 2966, 2872, 2097, 1343  $cm^{-1}$ . Anal. ( $C_{10}H_{14}NOS$ ), C, H, N, S.

**2-endo-Hydroxynorbornyl Isothiocyanate 28.** To a flask charged with 0.515 g (4.54 mmol) of 5-hydroxy-2-norbornene (mixture of *endo* and *exo*, Aldrich Chemical Co.) 0.530 g (5.45 mmol) of KSCN, and 8 mL of benzene was added a premixed solution of  $H_2SO_4$  (0.400 g) in 0.2 mL of  $H_2O$  at RT. After 3 days at 55 °C, the reaction mixture was filtered and chromatographed (40/60 ether/hexane) to give 0.075 g of product as a white solid in 10% yield. A portion of the material was recrystallized from hexane/ether for analysis (white flakes; mp 64–66 °C):  $^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  4.24–4.18 (m, 1H), 3.67–3.65 (m, 1H), 2.52

(ddd,  $J$  = 13.2, 7.6, 2.0 Hz, 1H), 2.41 (d,  $J$  = 5.2 Hz, 1H), 2.35 (td,  $J$  = 5.2, 1.6 Hz, 1H), 2.00 (ddd,  $J$  = 15.6, 10.4, 5.6 Hz, 1H), 1.69 (dq,  $J$  = 14.0, 3.2 Hz, 1H), 1.57–1.53 (m, 2H), 1.45–1.42 (m, 1H), 0.76 (dt,  $J$  = 14.0, 3.2 Hz, 1H); FT-IR ( $CHCl_3$ ) 3613, 3472, 2966, 2097, 1343  $cm^{-1}$ . Anal. ( $C_6H_{11}NOS$ ) C, H, N, S.

**Bioassay Procedures. Measurement of Inducer Potency in Hepta 1c1c7 Murine Hepatoma Cells.** These determinations were carried out on cells grown in 96-well microtiter plates according to minor modifications<sup>18</sup> of the procedure of Prochasko and Santamaria.<sup>4</sup> The cells (10 000 per well) were grown for 24 h in medium containing 10% heat- and charcoal-treated fetal calf serum and then exposed to serial dilutions of the inducers for 48 h before measurement of QR specific activity. Compounds were dissolved in acetonitrile and diluted so that the final concentration of solvent was 0.1% by volume in all wells.

**Induction of Quinone Reductase and Glutathione Transferase Activities in Mouse Tissues. Treatment of Animals.** Five-week-old female CD-1 mice (Charles River Laboratories, Wilmington, MA) were acclimated for 1 week on AIN 76A pellets diet. The animals were housed in plastic cages (four or five per cage). Each mouse received 0.1 mL of Emulphor EL620P (GAF, Linden, NJ) alone (10 mice) or 0.1 mL of Emulphor containing 7.5  $\mu$ mol (4 mice), 15  $\mu$ mol (5 mice), or 30  $\mu$ mol (5 mice) of exo-2-acetyl-6-isothiocyanatonorbornane, daily by gavage for 5 days. Twenty-four hours after the last treatment, the animals were killed by carbon dioxide inhalation, the organs were removed, frozen in liquid nitrogen, and stored at –80 °C until analysis.

**Preparation of Tissue Cytosols and Assay of Their Enzymatic Activities.** The cytosols were prepared as described.<sup>18,19</sup> In the present experiments the entire proximal small intestine (after removal of contents) was homogenized rather than the mucosal scrapings.

Specific enzyme activities were measured at 25 °C as described<sup>18,20</sup> except that the assay systems were miniaturized (to one-tenth volume) so that measurements could be made in 96-well microtiter plates with the use of a microtiter plate reader (UVmax, Molecular Devices, Palo Alto, CA). The QR specific activities were determined in a final volume of 0.3 mL by measuring the NADPH-dependent rate of menadiol-mediated reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 610 nm. The specific activities of GST were measured with DCNB and CDNB in final volumes of 0.2 mL at 340 nm. Suitable rates were obtained by use of appropriate volumes and dilutions of cytosols and were derived from absorbance changes during the initial 2 min, based on the average of four wells. Rates were corrected for absorbance changes in wells containing all components except cytosol. Protein determinations were made according to Bradford.<sup>21</sup> The ratios of the mean enzyme specific activities (nanomoles of product formed per minute per milligram of protein) of tissue cytosols from animals treated with the inducer to those receiving vehicle only were then calculated ( $\pm$  SEM). The standard errors of these ratios were calculated.<sup>18</sup>

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## A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure

(chemoprotection/enzyme induction/isothiocyanates/sulforaphane/quinone reductase)

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**ABSTRACT** Consumption of vegetables, especially crucifers, reduces the risk of developing cancer. Although the mechanisms of this protection are unclear, feeding of vegetables induces enzymes of xenobiotic metabolism and thereby accelerates the metabolic disposal of xenobiotics. Induction of phase II detoxication enzymes, such as quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2] and glutathione *S*-transferases (EC 2.5.1.18) in rodent tissues affords protection against carcinogens and other toxic electrophiles. To determine whether enzyme induction is responsible for the protective properties of vegetables in humans requires isolation of enzyme inducers from these sources. By monitoring quinone reductase induction in cultured murine hepatoma cells as the biological assay, we have isolated and identified (–)-1-isothiocyanato-(4*R*)-(methylsulfinyl)butane [ $\text{CH}_3\text{—SO—}(\text{CH}_2)_4\text{—NCS}$ , sulforaphane] as a major and very potent phase II enzyme inducer in SAGA broccoli (*Brassica oleracea italica*). Sulforaphane is a monofunctional inducer, like other anticarcinogenic isothiocyanates, and induces phase II enzymes selectively without the induction of aryl hydrocarbon receptor-dependent cytochromes P-450 (phase I enzymes). To elucidate the structural features responsible for the high inducer potency of sulforaphane, we synthesized racemic sulforaphane and analogues differing in the oxidation state of sulfur and the number of methylene groups:  $\text{CH}_3\text{—SO}_m\text{—}(\text{CH}_2)_n\text{—NCS}$ , where  $m = 0, 1, \text{ or } 2$  and  $n = 3, 4, \text{ or } 5$ , and measured their inducer potencies in murine hepatoma cells. Sulforaphane is the most potent inducer, and the presence of oxygen on sulfur enhances potency. Sulforaphane and its sulfide and sulfone analogues induced both quinone reductase and glutathione transferase activities in several mouse tissues. The induction of detoxication enzymes by sulforaphane may be a significant component of the anticarcinogenic action of broccoli.

Individuals who consume large amounts of green and yellow vegetables have a lower risk of developing cancer (1–3). Feeding of such vegetables to rodents also protects against chemical carcinogenesis (4, 5), and it results in the induction in many tissues of phase II enzymes—e.g., quinone reductase [QR; NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2] and glutathione *S*-transferases (EC 2.5.1.18) (11–17). Although much evidence suggests that induction of these enzymes is a major mechanism responsible for this protection (18–20), the precise role of enzyme induction in protection of humans requires clarification. The preceding report (21) shows that measurement of QR activity in Hepa 1c1c7 murine hepatoma cells provides a rapid, reliable, and convenient index of phase II enzyme inducer activity in vegetables. Using this assay (21–24), we found that cruciferous vegetables (broccoli, cauliflower, mustard, cress, brussels sprouts) were a rich source of inducer activity. We chose to investi-

gate broccoli (*Brassica oleracea italica*) specifically because it is consumed in substantial quantities by Western societies and has been shown to contain abundant phase II enzyme inducer activity (21). In this paper we describe the isolation and identification of a potent major phase II enzyme inducer from broccoli.

### MATERIALS AND METHODS

**Source of Vegetables and Preparation of Extracts.** SAGA broccoli was grown by Andrew Ayer (Maine Packers, Caribou, ME). SAGA is synonymous with Mariner broccoli (Peto Seed, Arroyo Grande, CA) and was adapted for growing in Maine by Smith, Ayer, Goughan, and Arrow. The broccoli was harvested when ripe, frozen immediately, shipped to our laboratory in dry ice, and stored at  $-20^\circ\text{C}$  until processed.

For preliminary survey of inducer activity in broccoli samples, florets were homogenized with 2 vol of water at  $4^\circ\text{C}$ , and the resultant soups were lyophilized to give powders, which were stored at  $-20^\circ\text{C}$ . Portions (400 mg) of these powders were extracted for 6 hr with 14 ml of acetonitrile in glass-stoppered vessels on a wrist-action shaker at  $4^\circ\text{C}$ . The extracts were filtered through a sintered glass funnel and evaporated to dryness in a rotating evaporator ( $<40^\circ\text{C}$ ). The residues were dissolved or suspended in 100  $\mu\text{l}$  of dimethyl formamide and assayed for inducer activity.

**Assay of Inducer Activity.** Inducer potency for QR was measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates (21, 24). The cells (10,000 per well) were grown for 24 hr and then exposed to inducer for 48 hr. Usually 20  $\mu\text{l}$  of the solutions to be assayed (in acetonitrile or dimethyl formamide) was added to 10.0 ml of medium and 2-fold serial dilutions were used for the microtiter plates. The final organic solvent concentration was always less than 0.2% by volume. One unit of inducer activity is defined as the amount that when added to a single microtiter well (containing 150  $\mu\text{l}$  of medium) doubles the QR specific activity. The inducer potency of compounds of known structure has been determined in the above system also, and it is expressed as

Abbreviations: QR, quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2]; CD value, the concentration of a compound required to double the quinone reductase specific activity in Hepa 1c1c7 murine hepatoma cells.

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Enzymes of xenobiotic metabolism belong to two families (6): (i) phase I enzymes (e.g., cytochromes P-450), which functionalize compounds, usually by oxidation or reduction; although their primary role is to detoxify xenobiotics, several cytochromes P-450 can activate procarcinogens to highly reactive ultimate carcinogens (7); and (ii) phase II enzymes, which conjugate functionalized products with endogenous ligands (e.g., glutathione, glucuronic acid, sulfate) and play primarily a detoxication role (8). QR is considered a phase II enzyme because it serves protective functions (9), is induced coordinately with other phase II enzymes, and is regulated by enhancer elements similar to those that regulate glutathione transferases (10).

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the concentration required to double (CD value) the QR activity.

The inductions of QR and glutathione transferase activities in mouse organs were studied according to a standard protocol (25).

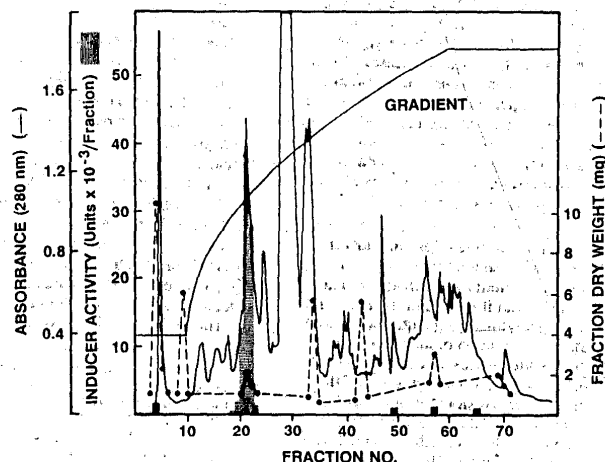
**Synthesis of Compounds.** (*R,S*)-Sulforaphane (CAS 4478-93-7) was prepared according to Schmid and Karrer (26) except that gaseous thiomethanol was replaced by sodium thiomethoxide. The sulfide analogues,  $\text{CH}_3\text{—S—(CH}_2\text{)}_n\text{—NCS}$ , where  $n$  is 4 [erucin (CAS 4430-36-8)] or 5 [berteroin (CAS 4430-42-6)] were prepared as described (27), and the three-carbon analogue [iberverin (CAS 505-79-3)] was prepared from phthalimidopropyl bromide (26). IR spectra of all three sulfide analogues showed strong absorptions near  $2150\text{ cm}^{-1}$ , characteristic of isothiocyanates.  $^1\text{H NMR}$  spectra of these compounds show sharp singlets at  $\delta\ 2.10\text{ ppm}$  ( $\text{CH}_3\text{—S}$  group). The sulfoxide analogues where  $n$  is 3 [iberin (CAS 505-44-2)] or 5 [alyssin (CAS 646-23-1)] were prepared by the same method as sulforaphane. IR spectra of these compounds showed strong absorptions near  $2100\text{ cm}^{-1}$ , assigned to the  $\text{—NCS}$  group.  $^1\text{H NMR}$  spectra also showed sharp singlets around  $\delta\ 2.5\text{ ppm}$ , consistent with the presence of the  $\text{CH}_3\text{—SO}$  group. The sulfone analogues,  $\text{CH}_3\text{—SO}_2\text{—(CH}_2\text{)}_n\text{—NCS}$ , where  $n$  is 3 [cheirolin (CAS 505-34-0)], 4 [erysolin (CAS 504-84-7)], or 5 (unreported) were prepared by known methods (28).  $^1\text{H NMR}$  ( $\delta\approx 2.9\text{ ppm}$ , for  $\text{CH}_3\text{—SO}_2\text{—}$ ) and IR spectra of these compounds were consistent with the structures. Every analogue except 1-isothiocyanato-5-methylsulfonylpentane [ $\text{CH}_3\text{—SO}_2\text{—(CH}_2\text{)}_5\text{—NCS}$ ] has been isolated from plants (29).

## RESULTS

**Isolation of Inducer Activity.** We selected SAGA broccoli for study because acetonitrile extracts of lyophilized homogenates of this variety were especially rich in inducer

activity (62,500 units per g) in comparison with other vegetables (21). Fractionation of acetonitrile extracts of SAGA broccoli by preparative reverse-phase HPLC (Fig. 1) with a water/methanol solvent gradient resulted in recovery of 70–90% of the applied inducer activity in the chromatographic fractions. Surprisingly, the majority (about 65–80% in several chromatographies) of the recovered activity was associated with a single and relatively sharp peak [fractions 18–23; eluted at 64–71% (vol/vol) methanol]. This HPLC procedure was therefore adopted as the first step of the larger-scale isolation of inducer activity.

Lyophilized SAGA broccoli was extracted three times with acetonitrile (35 ml/g) for 6 hr each at  $4^\circ\text{C}$ . The pooled extracts were filtered and evaporated to dryness under reduced pressure on a rotating evaporator ( $<40^\circ\text{C}$ ). About 1 g of residue from 640 g of fresh broccoli (64 g of lyophilized powder) contained  $3.6 \times 10^6$  units of inducer activity. The residue was mixed thoroughly with 120 ml of methanol/water (25/75, vol/vol) and the insoluble fraction was discarded. Although not all of the residue obtained from the extraction was soluble in aqueous methanol, the solvent partition procedure resulted in substantial purification without significant loss of inducer activity. Portions of the extract were dried in a vacuum centrifuge and dissolved in small volumes of dimethyl formamide (0.75–1.0 ml per 50 mg of residue), and 50-mg portions were subjected to HPLC (nine runs) as described in the legend of Fig. 1. Fractions 18–23 from all runs were pooled, evaporated to dryness, applied in acetonitrile to five preparative silica TLC plates ( $100 \times 200 \times 0.25\text{ mm}$ ), and developed with acetonitrile, which was run to the top of each plate three times. Four major fluorescence-quenching components were resolved, and nearly all (99%) of the inducer activity migrated at  $R_f\ 0.4$ . The active bands were eluted with acetonitrile, pooled, and fractionated by two runs on a second preparative reverse-phase HPLC in a water/



**FIG. 1.** Reverse-phase HPLC of acetonitrile extract of SAGA broccoli showing the distribution of absorbance at 280 nm; total inducer activity (units per fraction), and dry weight of each fraction. Lyophilized SAGA broccoli floret powder (16 g) was extracted three times (for 6 hr each) with 560-ml portions of acetonitrile on a shaker at  $4^\circ\text{C}$ . The extracts were filtered and evaporated to dryness on a rotating evaporator ( $<40^\circ\text{C}$ ). The residue (202 mg) was suspended in 3.0 ml of methanol and filtered successively through 0.45- and 0.22- $\mu\text{m}$  porosity filters. The insoluble material was discarded. The filtrate was assayed for total inducer activity, and a 0.75-ml (50.5-mg) aliquot of the methanol extract was subjected to HPLC on a reverse-phase preparative column (Whatman; Partisil 10 ODS-2;  $50 \times 1.0\text{ cm}$ ) equilibrated with methanol/water (30/70, vol/vol), eluted at a rate of 3.0 ml/min, and 6.0-ml fractions were collected. Elution solvent: 30 ml of initial solvent, followed by 330 ml of a convex gradient (Waters Gradient program 5) to 100% methanol, and then by 90 ml of 100% methanol. The fractions were evaporated on a vacuum centrifuge (Savant Speed-Vac Concentrator), and the residues were weighed, redissolved in 0.1 ml of dimethyl formamide, and assayed for inducer activity. The activity applied (0.75 ml = 104,000 units) was recovered principally in fractions 18–23 (84,600 units, 81%), and minor amounts of activity were found in fractions 4, 49, 57, and 65. The total recovery of inducer activity in all fractions was 90% of that applied to the column.

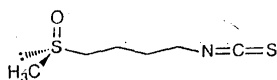


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acetonitrile gradient (Fig. 2). Ultraviolet absorption and inducer activity were eluted in a sharp coincident peak (at 66% acetonitrile) that contained all of the activity applied to the column. Evaporation (<40°C) of the active fractions gave 8.9 mg of a slightly yellow liquid, which contained 558,000 inducer units (overall yield 15%) and migrated as a single band on TLC.

**Identification of Inducer.** The identity of the inducer was established by spectroscopic methods and confirmed by chemical synthesis. It is (-)-1-isothiocyanato-(4*R*)-(methylsulfinyl)butane, known as sulforaphane or sulphoraphane (CAS 4478-93-7):



Sulforaphane has been synthesized (26) and isolated from leaves of hoary cress (30) and from other plants (31), and the absolute configuration has been assigned (32). The closely related olefin sulforaphene [4-isothiocyanato-(1*R*)-(methylsulfinyl)-1-(*E*)-butene (CAS 2404-46-8)] has been isolated from radish seeds and other plants (33, 34) and has also been synthesized (35, 36).

The following evidence establishes that (*R*)-sulforaphane is the inducer isolated from broccoli. UV spectrum (H<sub>2</sub>O):  $\lambda_{\text{max}}$  238 nm,  $\epsilon_{238}$  910 M<sup>-1</sup>cm<sup>-1</sup>; addition of NaOH (0.1 M) blue-shifted ( $\lambda_{\text{max}}$  226 nm) and intensified ( $\epsilon_{226}$  15,300 M<sup>-1</sup>cm<sup>-1</sup>) this absorption band, consistent with the behavior of isothiocyanates (37). IR (Fourier transform, neat): strong absorptions at 2179 and 2108 cm<sup>-1</sup> and also at 1350 cm<sup>-1</sup>, characteristic of isothiocyanates (27). <sup>1</sup>H NMR (400 MHz,

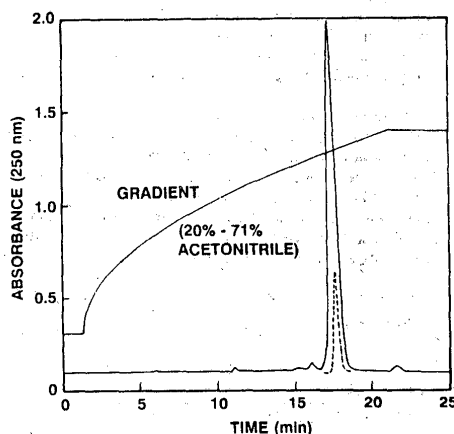
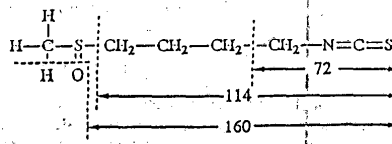


FIG. 2. Second reverse-phase preparative HPLC of enzyme inducer activity from SAGA broccoli. The active inducer bands obtained from two or three preparative silica TLC plates (see text) were combined, eluted with acetonitrile, filtered twice through 0.22- $\mu$ m porosity filters, and evaporated to dryness on a vacuum centrifuge. The residue was dissolved in 0.5 ml of acetonitrile and applied to a reverse-phase preparative HPLC column (Whatman; Partisil ODS-2; 50  $\times$  1.0 cm), which was developed with a convex gradient (Waters Gradient program 5) of acetonitrile/water from 20:80% to 71:29% (vol/vol) at a flow rate of 3.0 ml/min during a 20-min period. The eluate from 17.0 to 19.0 min was collected as a pool and assayed for inducer activity; 99% of the inducer activity was recovered in this pool. The elution position of (*R,S*)-sulforaphane is shown (---).

<sup>2</sup>HCl<sub>3</sub>):  $\delta$  3.60 (t, 2H,  $J$  = 6.1 Hz,  $-\text{CH}_2-\text{NCS}$ ), 2.80–2.66 (m, 2H,  $-\text{CH}_2-\text{SO}-$ ), 2.60 (s, 3H,  $\text{CH}_3-\text{SO}-$ ), and 1.99–1.86 ppm (m, 4H,  $-\text{CH}_2\text{CH}_2-$ ). <sup>13</sup>C NMR (400 MHz, C<sup>2</sup>HCl<sub>3</sub>):  $\delta$  53.5, 44.6, 38.7, 29.0, and 20.1 ppm. Mass spectrometry (fast atom bombardment; thioglycerol matrix) gave prominent peaks at 178 ( $M + H$ )<sup>+</sup> and 355 ( $M_2 + H$ )<sup>+</sup>. Electron impact mass spectrometry gave a small molecular ion ( $M^+$ ) at 177, and chemical ionization mass spectrometry gave a small molecular ion ( $M + H$ )<sup>+</sup> at 178 and prominent fragment ions with masses of 160, 114, and 72, consistent with the following fragmentation:



Precise masses of molecular and fragment ions obtained by electron impact mass spectrometry were 177.0286 (calculated for C<sub>6</sub>H<sub>11</sub>NS<sub>2</sub>, 177.0283), 160.0257 (calculated for C<sub>6</sub>H<sub>10</sub>NS<sub>2</sub>, 160.0255), and 71.9909 (calculated for C<sub>3</sub>H<sub>7</sub>NS<sub>1</sub>, 71.9908). In addition, for the mass 160 fragment, the peaks at 161 ( $M + 1$ ) and 162 ( $M + 2$ ) were 8.43% (calculated, 8.44%) and 9.45% (calculated, 10.2%), respectively, of the parent ion. Similarly, for the mass 72 fragment, the peaks at 73 ( $M + 1$ ) and 74 ( $M + 2$ ) were 3.42% (calculated, 3.32%) and 5.23% (calculated, 4.44%), respectively, of the parent ion. Hence the isotope compositions corrected for the natural isotope abundance (of <sup>13</sup>C, <sup>15</sup>N, <sup>33</sup>S, and <sup>34</sup>S) were consistent with the relative intensities of the  $M + 1$  and  $M + 2$  ions of both fragments. The optical rotation of the isolated material was  $[\alpha]_D^{25} -63.6^\circ$  ( $c$  = 0.5, CH<sub>2</sub>Cl<sub>2</sub>), thus establishing that the product is largely, if not exclusively, the (-)-(*R*) enantiomer (literature  $[\alpha]_D^{25} -79^\circ$ ,  $-73.2^\circ$ ,  $-66^\circ$ ; refs. 26, 30, and 38, respectively). The spectroscopic properties of synthetic (*R,S*)-sulforaphane were identical to those of the isolated product.

**Relation of Structure to Inducer Activity Among Sulforaphane Analogues.** To define the structural features of sulforaphane (chirality, state of oxidation of sulfur of the thiomethyl group, number of methylene bridging groups) important for inducer activity, we synthesized (*R,S*)-sulforaphane and the following analogues and measured their inducer potencies: CH<sub>3</sub>-S-(CH<sub>2</sub>)<sub>*n*</sub>-N=C=S ( $n$  = 3, 4, or 5); CH<sub>3</sub>-SO-(CH<sub>2</sub>)<sub>*n*</sub>-N=C=S ( $n$  = 3 or 5); and CH<sub>3</sub>-SO<sub>2</sub>-(CH<sub>2</sub>)<sub>*n*</sub>-N=C=S ( $n$  = 3, 4, or 5).

**Induction of QR in Murine Hepatoma Cells.** The chirality of the sulfoxide does not affect inducer potency, since isolated (*R*)-sulforaphane and synthetic (*R,S*)-sulforaphane gave closely similar CD values of 0.4–0.8  $\mu$ M. Sulforaphane is therefore the most potent monofunctional (see below) inducer that has been identified (19, 20). Both (*R*)- and (*R,S*)-sulforaphane were relatively noncytotoxic; the concentrations required to depress cell growth to one-half were 18  $\mu$ M.

Sulforaphane and the corresponding sulfone (erysolin) were equipotent as inducers of QR, whereas the corresponding sulfide (erucin) was about one-third as active (Table 1). Oxidation of the side-chain sulfide to sulfoxide or sulfone enhanced inducer potency, and compounds with 4 or 5 methylene groups in the bridge linking CH<sub>3</sub>- and  $-\text{N}=\text{C}=\text{S}$  were more potent than those with 3 methylene groups (Table 1).

Mutants of Hepa 1c1c7 cells defective in the Ah (aryl hydrocarbon) receptor, or expression of cytochrome P-450IA1 can distinguish monofunctional inducers (which induce phase II enzymes selectively) from bifunctional in-



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Table 1. Potency of induction of QR in Hepa 1c1c7 cells by sulforaphane and analogues

Compound	CD value, $\mu\text{M}$		
	$n = 3$	$n = 4$	$n = 5$
$\text{CH}_3\text{---S---}(\text{CH}_2)_n\text{---N=C=S}$	3.5 (Iberverin)	2.3 (Erucin)	1.7 (Berteroin)
$\text{CH}_3\text{---S---}(\text{CH}_2)_n\text{---N=C=S}$	2.4 (Iberin)	0.4-0.8 (Sulforaphane)	0.95 (Alyssin)
$\text{CH}_3\text{---S---}(\text{CH}_2)_n\text{---N=C=S}$	1.3 (Cheirolin)	0.82 (Erysolin)	0.98

Trivial names are given in parentheses. See Kjær (29).

ducers (which elevate both phase I and II enzymes) (39, 40). When sulforaphane was tested with the BP<sub>1</sub>c1 mutant (41) (defective in transport of the liganded Ah receptor to the nucleus), and the c1 mutant (42) (which synthesizes inactive cytochrome P-450IA1), induction of QR was normal (data not shown). Sulforaphane is, therefore, like benzyl isothiocyanate, a monofunctional inducer (40) and is unlikely to elevate activities of cytochromes P-450 that could activate carcinogens.

**Induction of QR and Glutathione Transferase Activities in Mice.** When synthetic (*R,S*)-sulforaphane, erysolin, and erucin were administered to female CD-1 mice by gavage (25), induction of QR and glutathione transferase activities was observed in the cytosols of several organs (Table 2). Sulforaphane and erucin (in daily doses of 15  $\mu\text{mol}$  for 5 days) raised both enzyme activities 1.6- to 3.1-fold in liver, forestomach, glandular stomach, and mucosa of proximal small intestine, and to a lesser degree in lung. The sulfone (erysolin) was more toxic, but even 5- $\mu\text{mol}$  daily doses for 5 days elevated the specific activities of these enzymes in some tissues examined. We therefore conclude that sulforaphane and its analogues not only induce QR in Hepa 1c1c7 murine hepatoma cells but also induce both QR and glutathione transferase activities in a number of murine organs.

### DISCUSSION

Two considerations prompt the belief that sulforaphane is a major and probably the principal inducer of phase II enzymes present in extracts of SAGA broccoli. First, high yields of

inducer activity were obtained at each step of the isolation, and even in the first HPLC (Fig. 1) more than 60% of the inducer activity was contained in a single chromatographic peak, the biological activity of which could not be subfractionated. Second, when a totally independent method of isolation of inducer activity by high-vacuum sublimation of lyophilized broccoli (5  $\mu\text{m}$  Hg pressure, 60-165°C, condenser at -15°C) was used, nearly all the isolated inducer activity was found in the methanol-soluble portion of the sublimate. Moreover, on HPLC (Fig. 2) this sublimed material gave rise to only a single isothiocyanate-containing fraction, which on TLC comigrated with authentic sulforaphane and after further purification by TLC provided a high yield of sulforaphane characterized unequivocally by NMR.

The finding that the majority of the inducer activity of SAGA broccoli probably resides in a single chemical entity, an isothiocyanate, is of considerable interest. Isothiocyanates (mustard oils) and their glucosinolate precursors are widely distributed in higher plants and are especially prevalent among cruciferous vegetables (29). Sulforaphane has been identified in species of *Brassica*, *Erica*, and *Iberis* (29, 31).

Isothiocyanates have been shown to block chemical carcinogenesis. In rats, 1-naphthyl isothiocyanate suppressed hepatoma formation by 3-methylcholanthrene, 2-acetylaminofluorene, diethylnitrosamine, *m*-toluenediamine, and azo dyes (43-46). In mice, benzyl isothiocyanate blocked the neoplastic effects of diethylnitrosamine or benzo[*a*]pyrene on lung and forestomach (47, 48), and a variety of phenylalkyl isothiocyanates reduced the pulmonary carcinogenicity of

Table 2. Induction of QR and glutathione *S*-transferase (GST) in mouse tissues by sulforaphane and analogues

Inducer	Dose, $\mu\text{mol}$ per mouse per day	Enzyme	Ratio of specific activities (treated/control)				
			Liver	Forestomach	Glandular stomach	Proximal small intestine	Lung
$\text{CH}_3\text{---S---}(\text{CH}_2)_4\text{---NCS}$ Erucin	15	QR	2.19 $\pm$ 0.06	1.64 $\pm$ 0.18*	1.72 $\pm$ 0.11	3.10 $\pm$ 0.20	1.66 $\pm$ 0.13
		GST	1.86 $\pm$ 0.08	2.51 $\pm$ 0.11	2.07 $\pm$ 0.08	3.00 $\pm$ 0.21	1.41 $\pm$ 0.11*
$\text{CH}_3\text{---S---}(\text{CH}_2)_4\text{---NCS}$ Sulforaphane	15	QR	2.45 $\pm$ 0.07	1.70 $\pm$ 0.18*	2.35 $\pm$ 0.06	2.34 $\pm$ 0.19	1.37 $\pm$ 0.14*
		GST	1.86 $\pm$ 0.08	1.98 $\pm$ 0.08	2.97 $\pm$ 0.08	2.13 $\pm$ 0.20	1.17 $\pm$ 0.09†
$\text{CH}_3\text{---S---}(\text{CH}_2)_4\text{---NCS}$ Erysolin	5	QR	1.62 $\pm$ 0.09	1.05 $\pm$ 0.21†	1.57 $\pm$ 0.08†	1.22 $\pm$ 0.20†	1.00 $\pm$ 0.11†
		GST	1.08 $\pm$ 0.11†	1.45 $\pm$ 0.15†	1.94 $\pm$ 0.10†	0.87 $\pm$ 0.20†	1.09 $\pm$ 0.13†

The compounds were administered to 6-week-old female CD-1 mice (4 or 5 mice per group) by gavage in indicated single daily doses in 0.1 ml of Emulphor EL 620P (GAF; Linden, NJ) for 5 days. Cytosols were prepared from the tissues 24 hr after the last treatment and assayed for enzyme activities (glutathione *S*-transferase was measured with 1-chloro-2,4-dinitrobenzene). The specific activities (nmol·min<sup>-1</sup>·mg<sup>-1</sup> SEM) of organs of vehicle-treated control mice were as follows: Liver: QR, 47  $\pm$  0.70; GST, 1014  $\pm$  69. Forestomach: QR, 1038  $\pm$  155; GST, 1182  $\pm$  74. Glandular stomach: QR, 3274  $\pm$  85; GST, 1092  $\pm$  81. Small intestine: QR, 664  $\pm$  119; GST, 1372  $\pm$  266. Lung: QR, 54  $\pm$  5.8; GST, 439  $\pm$  34. Data are presented as mean  $\pm$  SEM. All ratios were significantly different from 1.0 with  $P < 0.01$ , except for \*,  $P < 0.05$ , and †,  $P > 0.05$ .

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the tobacco-derived carcinogenic nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (49, 50). The anticarcinogenic effects of previously studied isothiocyanates may be related to their capacity to induce phase II enzymes, which are involved in the metabolism of carcinogens (51-57).

It will be important to establish whether the alterations of drug metabolism observed in humans and rodents after the ingestion of cruciferous vegetables (58, 59) can be ascribed to their content of sulforaphane. The finding that this isothiocyanate is a major monofunctional inducer of phase II enzymes in broccoli also provides the possibility of clarifying the relationship between enzyme induction and chemoprotection.

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Medical Sciences

## Rapid detection of inducers of enzymes that protect against carcinogens

(cancer/chemoprotection/enzyme induction/quinone reductase/vegetables)

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**ABSTRACT** Dietary composition is a major determinant of cancer risk in humans and experimental animals. Major and minor components of the diet may enhance or suppress the development of malignancy. Many dietary constituents also modify the metabolism of carcinogens by induction of enzymes involved in xenobiotic metabolism, and this is one well-established mechanism for modulating the risk of cancer. We have developed a simple system for rapid detection and measurement of the induction of enzymes that detoxify carcinogens (phase II enzymes), based on the direct assay of the activity of quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2] in murine hepatoma cells grown in microtiter plate wells. Survey of extracts of a variety of commonly consumed, organically grown vegetables for quinone reductase inducer activity identified crucifers (and particularly those of the genus *Brassica*) as singularly rich sources. It is therefore of interest that high consumption of these types of vegetables has been correlated with decreased cancer risk in humans. The assay system also measures toxicity, which was unrelated to inducer potency among the vegetable extracts examined. By use of mutant hepatoma cells (defective in regulation of certain cytochrome P-450 enzymes) selective (monofunctional) inducers of protective phase II enzymes can be distinguished from (bifunctional) inducers that also elevate cytochromes P-450 (phase I enzymes) and thereby pose the risk of carcinogen activation. The assay system therefore permits not only rapid detection of inducers of anticarcinogenic enzymes in the human diet but also elucidation of effects of storage and processing on inducer activities.

Extrinsic factors, including personal life-styles, play a major role in the development of most human malignancies (1-3). Cigarette smoking and consumption of alcohol, exposure to synthetic and naturally occurring carcinogens, radiation, drugs, infectious agents, and reproductive and behavioral practices are now widely recognized as important contributors to the etiology of cancer. But perhaps most surprising is the inference that normal human diets play causative roles in more than one-third (and possibly even two-thirds) of human neoplasia (1-3). Our food contains not only numerous mutagens and carcinogens but also a variety of chemicals that block carcinogenesis in animal models (4-11). Furthermore, carcinogens can even protect against their own toxic and neoplastic effects or those of other carcinogens—i.e., carcinogens may act as anticarcinogens (12-14). Clearly, dietary modifications modulate cancer risk in various ways: for instance, through changes in caloric intake, by altering the consumption of nutritive and nonnutritive major components, and by providing exposure to numerous minor chemicals that may be genotoxic or protective (4-7, 9-11, 15-19). Rational recommendations for modifying human diets to

reduce the risk of cancer require identification of dietary carcinogens and chemoprotectors, even though interactions among such factors in the etiology of cancer are complex (20). Whereas extensive efforts have been made to identify dietary carcinogens and mutagens (4-6), chemoprotective components have received far less attention. This paper describes a method for detecting and identifying anticarcinogenic components in human diets.

Since a major mechanism regulating neoplasia is the balance between phase I enzymes, which activate carcinogens, and phase II enzymes<sup>‡</sup> (25, 26), which detoxify them, we have developed a cell culture system for simple and rapid detection of dietary components that enhance phase II detoxication enzymes. With this procedure we surveyed extracts of a variety of vegetables for their ability to induce such protective enzymes. In the accompanying paper (27) we describe use of this method to isolate and identify a major inducer of protective enzymes from broccoli.

We chose vegetables as sources of inducers of detoxication enzymes for the following reasons. First, numerous epidemiological studies suggest that high consumption of yellow and green vegetables, especially those of the family Cruciferae (mustards) and the genus *Brassica* (cauliflower, cress, brussels sprouts, cabbage, broccoli), reduces the risk of developing cancer of various organs (28-34). Moreover, administration of vegetables or of some of their chemical components to rodents also protects against chemical carcinogenesis (9-11, 35). Second, well-documented evidence established that feeding of certain vegetables (e.g., brussels sprouts and cabbage) induces both phase I and phase II enzymes in animal tissues (36-44) and stimulates the metabolism of drugs in humans (36, 45, 46). The elevations of enzymes that metabolize xenobiotics may be highly relevant to the protective effects of vegetables, since relatively modest dietary changes not only affected the metabolism of drugs (44) but also modified the ability of carcinogens to cause tumors in rodents (15-19, 47-49).

Several lines of evidence provide compelling support for the proposition that induction of enzymes of xenobiotic

Abbreviation: QR, quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2].

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‡Enzymes of xenobiotic metabolism belong to two families: (i) phase I enzymes (cytochromes P-450), which functionalize compounds, usually by oxidation or reduction; although their primary role is to detoxify xenobiotics, several cytochromes P-450 can activate procarcinogens to highly reactive ultimate carcinogens (21); and (ii) phase II enzymes, which conjugate functionalized products with endogenous ligands (e.g., glutathione, glucuronic acid, sulfate) and serve primarily a detoxication role (22). Quinone reductase (QR) is considered a phase II enzyme because it has protective functions (23), is induced coordinately with other phase II enzymes, and is regulated by enhancer elements similar to those that control glutathione transferase (24).

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metabolism, and particularly phase II enzymes, results in protection against the toxic and neoplastic effects of carcinogens (25, 26): (i) Many seemingly unrelated compounds (including phenolic antioxidants, coumarins, cinnamates, 1,2-dithiole-3-thiones, isothiocyanates, lactones, thiocarbamates) can protect rodents against the effects of carcinogens under conditions that invariably evoke the induction of phase II enzymes in many tissues. Indeed, novel anticarcinogens have been isolated and identified solely on the basis of their ability to induce phase II enzymes (50, 51). (ii) Such anticarcinogens alter the metabolism of carcinogens and decrease the formation of mutagenic metabolites (52-54). (iii) Chemoprotection requires protein synthesis and is most effective if it precedes carcinogen challenge (12-14). (iv) Inducers of anticarcinogenic enzymes protect against a wide variety of structurally dissimilar carcinogens, suggesting the involvement of mechanisms that are not structurally fastidious, such as xenobiotic metabolism. (v) The enzymes that are elevated, e.g., glutathione transferases, quinone reduc-

tase [QR; NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2], UDP-glucuronosyltransferases, protect against the toxicities of electrophiles such as ultimate carcinogens. (vi) Cells in which glutathione transferases are elevated (by development of resistance to alkylating chemotherapeutic agents or by transfection with cloned enzymes) show decreased susceptibility to the toxicity of carcinogenic electrophiles and reduced formation of DNA adducts (55-57).

Resolution of the issue whether the anticarcinogenic effects of vegetables are mediated through the induction of enzymes of xenobiotic metabolism requires the systematic bioassay of these plants for inducer activity. Since measurement of enzyme induction in animals is laborious and expensive, we developed a simple screening procedure in which the specific activity of QR, a phase II enzyme, is measured in Hepa 1c1c7 murine hepatoma cells (58, 59). The feasibility of measuring inducer activity directly in cells grown in 96-well microtiter plates has simplified and accelerated the procedure (60), and the use of heat- and charcoal-treated serum in-

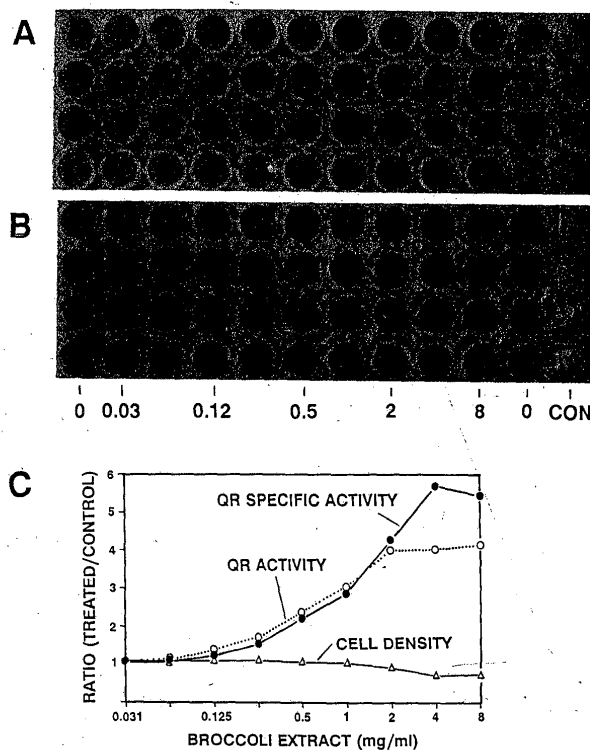


Fig. 1. Induction of QR in murine hepatoma cells by extracts of broccoli. (A and B) Photographs of sections of 96-well microtiter plates showing the induction of QR (A) and the cell density (B). (C) Graph showing the analysis of absorbances obtained from the plates. The assays were carried out on Hepa 1c1c7 murine hepatoma cells grown in microtiter plate wells and induced with serial 2-fold dilutions of acetonitrile extracts of lyophilized broccoli (Effe May). Details of the procedures are given in *Materials and Methods*. (A) The QR activities were measured in cell lysates by reduction of a tetrazolium dye. Note that the color (blue-brown) increases in intensity with the concentration of extract, indicating QR induction. (B) A parallel plate treated with the same dilutions of broccoli extract. The cells were stained with crystal violet. Note that there is a slight decrease in cell density at the highest concentrations (4-8 mg/ml), indicating mild cytotoxicity. (C) Graphical analysis of optical density information obtained from the above plates scanned at 610 nm (QR assay) and 490 nm (crystal violet assay) related to control wells that received the equivalent volume of acetonitrile only (0.2%). The total and specific activities of QR and the cell densities, expressed as ratios (treated/control), are shown on the ordinate. The concentrations of broccoli extract, shown below the designated microtiter plate wells and on the abscissa of the graph, are expressed as the amount of extract obtained from a given dry weight of broccoli (mg) added to each ml of culture medium (0-8 mg/ml). The QR activity and crystal violet density are related to cells that did not receive inducer. The columns of wells designated 0 mg/ml received no broccoli extract. CON designates the wells that contained no cells and served as the optical controls.



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creased its sensitivity (61). These cells respond to nearly all compounds that induce phase II enzymes (e.g., QR and glutathione transferases) in rodents, and conversely induction of QR in these cells is a reliable predictor of inducer activity in various rodent organs *in vivo* (27, 61–63).

### MATERIALS AND METHODS

**Sources of Vegetables and Preparation of Extracts.** All vegetables were grown under organic conditions without pesticides or artificial fertilizers that might contain enzyme inducers. They were stored at  $-20^{\circ}\text{C}$  after arrival in our laboratory, although the intervening storage history of some vegetables is not known. Vegetables were homogenized with 2 vol of cold water in a Waring Blender for 2 min at  $4^{\circ}\text{C}$ . The resultant soups were lyophilized to give dry powders, which were stored at  $-20^{\circ}\text{C}$ . Portions (400 mg) of these powders were extracted for 6–24 hr with 14 ml of acetonitrile by shaking in glass vessels at  $4^{\circ}\text{C}$ . The extracts were filtered through  $0.45\ \mu\text{m}$  porosity organic solvent-resistant filters and evaporated to dryness either in a vacuum centrifuge (Speed-Vac; Savant) or on a rotating evaporator ( $<40^{\circ}\text{C}$ ). The residues were dissolved or suspended in  $100\ \mu\text{l}$  of acetonitrile.

**Assay of Inducer Potency.** Inducer activity was measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates (60, 61). Duplicate plates were prepared. In a typical experiment 10,000 Hepa 1c1c7 cells were introduced into each well initially, grown for 24 hr, and then induced for 48 hr by exposure to medium containing serial dilutions of the extracts (or compounds) to be assayed. Usually  $20\ \mu\text{l}$  of the acetonitrile solutions to be assayed were added to  $10.0\ \text{ml}$  of medium and 2-fold serial dilutions were made in the microtiter plates so that the final volume in each well was  $150\ \mu\text{l}$  and the organic solvent concentration was 0.2%. QR activity (based on the formation of the blue-brown reduced tetrazolium dye) was measured with an optical microtiter plate scanner in cell lysates prepared in one plate, and the cell density was determined in the second plate by staining with crystal violet. Quantitative information on specific activity of QR, the inducer potency, and the cytotoxicity of the extract or compound tested is obtained by computer analysis of the absorbances (see Fig. 1). One unit of inducer activity is defined as the amount that when added to a single microtiter well doubled the QR specific activity.

### RESULTS AND DISCUSSION

Fig. 1 illustrates the measurement of inducer potency of extracts of organically grown broccoli (Effie May variety). The specific activities of QR were raised nearly 6-fold at the highest extract concentrations tested, at which less than 20% cytotoxicity was observed. The inductions obtained with broccoli (Fig. 1) and with other vegetable extracts (Fig. 2) were proportional to the quantity of extract added over a reasonably wide range. The toxicities of these extracts were modest and were unrelated to their inducer potencies (Fig. 2).

Extracts of a series of organically grown vegetables cultivated under a variety of conditions showed large differences in inducer potencies (Table 1). Because the dry weight content of the vegetables varied considerably, from 3.6% for a sample of bok choy to 26.5% for red leaf lettuce (mean 10% for 24 vegetables) (Table 1), we express the inducer activity of an extract in terms of the dry weight of vegetable yielding, upon extraction under standardized conditions, a given amount of inducer activity. This provides a measure of inducer potency, expressed as units per g of dry vegetable weight (see *Materials and Methods* for definition of unit). Although many vegetable extracts induced QR, certain families were consistently more potent inducers. For example,

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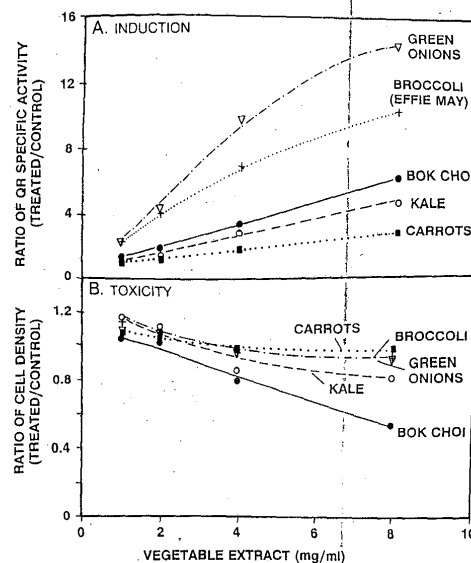


FIG. 2. Potency of induction of QR and toxicity of acetonitrile extracts obtained from five organically grown lyophilized vegetables (green onion, broccoli, bok choy, kale, and carrots) measured by the microtiter plate assay in Hepa 1c1c7 murine hepatoma cells. The extracts were prepared and assayed as described in Fig. 1 legend and *Materials and Methods*. The concentrations of extracts are expressed as the amount of extract per ml of culture medium derived from the indicated weight of dried vegetable. (A) Ratio of the specific activities of QR of treated to control cells. (B) Relative cell densities as determined by crystal violet staining measured at 490 nm. Note that the inductions are reasonably proportional to the amount of extract at lower induction ratios and that the inducer potencies and toxicities (which do not exceed 20% except in the case of bok choy) are not correlated.

whereas extracts of several Cruciferae had potent inducer activity, extracts of Solanaceae (peppers, potatoes, tomatoes) had low inducer activity. Of the 24 vegetables examined only 6 showed detectable toxicity; the others were nontoxic at the highest concentrations tested. Thus cytotoxicity of 20% was observed for red leaf lettuce at  $8.0\ \text{mg/ml}$ , for beets, cauliflower, and bok choy at  $4.0\ \text{mg/ml}$ , and for leeks and ginger at  $2.0\ \text{mg/ml}$ .

Cytotoxicity measurements are important because phase II enzyme inducers may be toxic and/or carcinogenic. Moreover, by use of mutant Hepa cells defective in aryl hydrocarbon receptor or cytochrome P-450 function (27, 63, 64), our assay system can distinguish *monofunctional* inducers (which elevate phase II enzymes selectively), from *bifunctional* inducers (which elevate both phase I and phase II enzymes) (63). Such information is crucial for identification of chemoprotective enzyme inducers for potential use in humans. Ideally such inducers should be monofunctional, because elevated activities of phase I enzymes may lead to carcinogen activation.

Since some crucifers (broccoli, brussels sprouts, cauliflower, cabbage) are consumed in substantial quantities in Western diets and are believed to protect against cancer, we examined the relation of inducer potency to variety, strain, location of growth, time of sowing, and time of harvest (Table 2). Although systematic examination of these factors under field conditions would require extensive studies over several



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Table 1. Potency of induction of QR in Hepa 1c1c7 murine hepatoma cells by acetonitrile extracts of representative samples of various vegetable families and species

Vegetable	Dry weight, %	Potency of QR induction, units*/g
Family Species/variety		
Chenopodiaceae Beets	17.3	<833
Spinach	6.8	1,280
Compositae Red leaf lettuce	26.5	3,030
Cruciferae Cauliflower	9.8	2,220
Bok choy	3.6	3,170
Broccoli (Effie May)	10.2	16,700
Broccoli (Winchester)	8.0	2,380
Green cabbage	9.4	1,550
Kale	9.2	2,220
Radish	3.7	1,040
Cucurbitaceae Zucchini	5.5	<833
Leguminosae Green beans	6.7	2,150
Sugar snap peas	14.5	<833
Liliaceae Asparagus	5.6	1,110
Green onions	5.1	22,200
Leeks	8.3	2,780
Rosaceae Apple	13.1	Inactive
Solanaceae Green peppers	6.4	Inactive
Red potatoes	15.7	Inactive
Sweet potatoes	20.2	Inactive
Tomatoes	6.2	<833
Umbelliferae Carrots	10.8	1,230 *
Celery	4.5	1,630
Zingiberaceae Ginger	13.1	4,440

\*One unit of inducer activity is defined as the amount of inducer required to double the QR specific activity of Hepa 1c1c7 cells growing in a microtiter well containing 150  $\mu$ l of medium. An entry of <833 units/g indicates that at the highest concentration tested (extract from 1.2 mg of dry vegetable/150  $\mu$ l medium) the QR specific activity was significantly elevated but not doubled. Inactive indicates less than 20% elevation of QR specific activity at highest concentration tested; extract from 1.2 mg of dry weight per 150  $\mu$ l of medium.

years of cultivation, it was important to determine whether such variables significantly affected the inducer activity. Except for a sample of kohlrabi, Cruciferae belonging to the species *Brassica oleracea* consistently and potentially induced QR (Table 2), with broccoli and brussels sprouts generally the most potent inducers. The inductive capacity of most crucifers appears to be independent of geographic location of growth and time of harvest, although late sowing may have enhanced modestly the potency of the induction. On the basis of these results a particular variety of broccoli (SAGA) was selected for isolation and identification of monofunctional inducer activity as described in the accompanying paper (27).

In summary, epidemiological studies point to the inverse relationship between vegetable consumption and the risk of epithelial cancer, and they suggest a practical approach to achieving protection by emphasizing that the typical Western diet is low in fruits and vegetables (20). A striking but perhaps not surprising conclusion is that the microtiter plate assay for induction of QR identifies the same vegetables (crucifers) that display protective properties *in vivo* (9-11, 28-35). It is critical to our understanding of the relationship of diet to cancer, however, that we assess dietary constituents not only for their abilities to induce anticarcinogenic enzymes but also for their toxic and carcinogenic properties. The simple and rapid assay can also determine the toxicity of extracts and, by use of appropriate mutant cells, distinguish monofunctional inducers from less desirable bifunctional ones. Moreover, the assay of phase II enzymes makes possible further detailed analysis of the effects of treatment of vegetables (e.g.,

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Table 2. Potency of induction of QR in Hepa 1c1c7 murine hepatoma cells by acetonitrile extracts of various cruciferous vegetables of the species *Brassica oleracea*

Vegetable ( <i>B. oleracea</i> subspecies and variety)	Dry weight, %	Potency of QR induction, units/g
Broccoli ( <i>B. oleracea italica</i> )		
Florets		
Emperor	9.6	6,670
Emperor (late sowing)	9.9	22,200
Green Valiant	10.4	16,700
Green Valiant (late sowing)	8.8	16,700
SAGA (older sample)	6.8	4,170
SAGA (younger sample)	8.0	11,100
SAGA (late sowing)	10.1	33,300
Violet Queen	9.6	3,700
Leaves only		
Emperor	7.0	5,560
SAGA (older sample)	9.4	3,030
SAGA (younger sample)	10.0	16,700
Violet Queen	10.6	7,410
Brussels sprouts ( <i>B. oleracea gemnifera</i> )		
Jade Cross E	10.8	11,100
Oliver	15.6	6,060
Teal	14.0	11,100
Green cabbage ( <i>B. oleracea capitata</i> )		
Nagoda 50	4.6	2,560
Perfect Ball	7.6	5,560
Primax	5.6	2,080
Red cabbage ( <i>B. oleracea capitata</i> )		
Lasso Red	11.2	13,300
Ruby Perfection	7.0	4,760
Cauliflower ( <i>B. oleracea botrytis</i> )		
Florets		
Andes	8.8	5,560
Montano	8.0	3,700
Leaves only		
Montano	7.4	3,330
Snow Crown	6.6	2,780
Kale ( <i>B. oleracea acephala</i> )		
Konserva	10.4	3,170
Winterbor	8.4	4,760
Winterbor (late sowing)	15.4	16,700
Kohlrabi ( <i>B. oleracea gongyloides</i> )		
Capri	5.2	1,330
Kolpak (late sowing)	6.0	1,590

The assays were performed as described in the text and legend to Table 1.

growth, storage, and cooking conditions) that might enhance or depress such induction, and also more definitive examination of the relationship of induction to chemoprotection.

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